

THE JOURNAL
OF
PHARMACOLOGY
AND
EXPERIMENTAL THERAPEUTICS

FOUNDED BY JOHN J. ABEL

OFFICIAL PUBLICATION
OF THE AMERICAN SOCIETY FOR PHARMACOLOGY AND
EXPERIMENTAL THERAPEUTICS INCORPORATED

Edited for the Society by

McK. CATTELL
E. M. K. GEILINO

V. E. HENDERSON
P. D. LAMSON
WM. DEB. MACNIDER

E. K. MARSHALL, JR.
CARL F. SCHMIDT

In Association with

THE BRITISH PHARMACOLOGICAL SOCIETY

Represented by

J. A. GUNN

SIR HENRY H. DALE

J. H. BURN

VOLUME 81
1944

BALTIMORE, MARYLAND

Reprinted by permission of The Williams and Wilkins Company, Baltimore, Md.

JOHNSON REPRINT CORPORATION
111 Fifth Avenue, New York 3, New York

Johnson Reprint Company Limited
Berkeley Square House, London, W. 1.

First reprinting, 1963, Johnson Reprint Corporation

CONTENTS

NUMBER 1, MAY, 1944

I	The Absorption and Excretion of Allantoin in Mammals	E Gordon Young, Helen P Wentworth and W W Hawkins	1
II	The Role of Pyrogens in the Alleged Leukocytic Response to Allantoin	E Gordon Young and W W Hawkins	10
III	The Toxicity of Sulfamerazine and Sulfamethazine	L H Schmidt, Hettie B Hughes, Elizabeth A Badger and Ida G Schmidt	17
IV	The Chemotherapeutic Activities of Sulfamerazine and Sulfamethazine	L H Schmidt, Clara L Sesler and Hettie B Hughes	43
V	The Effects of Sulfanilamide and Azide on Oxygen Consumption and Cell Division in the Egg of the Sea Urchin, <i>Arbacia punctulata</i>	Kenneth C Fisher, R J Henry and E Low	58
VI	Inhibition of Cholinesterase Activity of Nervous Tissues by Eseriac in vivo	G L Cantoni and O Loewi	67
VII	Anesthetic Activity of the <i>cis trans</i> Isomers of Trichloroethylides	Glycerol Thomas C Butler	72
VIII	Effect of Tyrosinase on Phenethylamine Derivatives	Lowell O Randall and George H Hitchings	77
IX	Amides, Amines and Related Compounds as Diuretics	Werner L Lipschitz and Zareh Hadidian	84
X	Cardiac and Blood Pressure Effects of Pitocin (Oxytocin) in Man	R A Woodbury, W F Hamilton, Perry P Valpitta, B E Abreu and H T Harper, Jr	95

NUMBER 2, JUNE, 1944

XI	Treatment of Experimental Renal Hypertension with Renal Extracts	G E Winkler, C A Johnson, W G Moss and M L Goldberg	101
XII	The Adrenolytic and Sympatholytic Actions of Yohimbine and Ethyl Yohimbine	Frederick F Yonkman, Don Stilwell and Robert Jeremias	111
XIII	Toxicological Studies of Phthalylsulfatbinzole	Paul A Mattis, Wilbur M Benson and Ethel S Koelle	116
XIV	Inhibitory Effect of Sulfonamides on the Action of Nicotine in the Isolated Intestine	E P Pick, G W Brooks and K Unna	133
XV	The Toxicity and Trepoaemicidal Activity of Amide Substituted Phenyl Arsenoxides and Their Derivatives	Harry Eagle, Ralph B Hogan, George O Doak and Harry G Steinman	142
XVI	Pharmacology and Chemistry of Substances with Cardiac Activity III The Effect of Simple Unsaturated Lactones and <i>t</i> Butyl Hydrogen Peroxide on the Isolated Frog Heart	Rafael Mendez	151
XVII	Plasma Concentrations Following the Oral Administration of Single Doses of the Principal Alkaloids of Cinchona Bark	Edwin P Hiatt	160
XVIII	Studies on Shock Induced by Hemorrhage VII The Destruction of Cozymase and Alloxazine Adenine Dinucleotide in Tissues during Shock	Margaret E Greig	164
XIX	The Antispasmodic Activity of Some 4 Morpholinealkyl Esters I Toxicity, Isolated Smooth Muscle Effects and Spasmodic Activity on the Ileum of Anesthetized Dogs	Harold F Chase, Arnold J Lehman and Frederick F Yonkman	174
XX	The Comparative Anoxic Effects from Carbon Monoxide Hemoglobin and Methemoglobin	David Lester and Leon A Greenberg	182

XXI. Chemotherapy of Filariasis in the Cotton Rat by Administration of Neostam and of Neostibosan. James T. Culbertson and Harry M. Rose..	189
XXII. Relationship of Chemical Structure of Sympathomimetic Amines to Ventricular Tachycardia during Cyclopropane Anesthesia. O. S. Orth, J. W. Stutzman and Walter J. Meek	197
XXIII. An Evaluation of the Influence of Succinate and Malonate on Barbiturate Hypnosis. Karl H. Beyer and Albert R. Latven.....	203

NUMBER 3, JULY, 1944

XXIV. Local Nervous Tissue Changes Following Spinal Anesthesia in Experimental Animals. Co Tui, M.D., A. L. Preiss, M. D., I. Baroham, M.D., and Marshall I. Nevin, M.D..	209
XXV. A Distribution Method for the Differentiation of Urinary Excretion Products of the Sulfonamide Drugs and the Role of These Products in Urolithiasis. John V. Soudi and Viola C. Jelinek.	218
XXVI. The Toxicity and Trypanocidal Activity of Some Organic Antimonials. L. G. Goodwin.....	224
XXVII. A Contribution to the Pharmacology of the Aliphatic Amines. Raymond P. Ahlquist.....	235
XXVIII. Studies on Shock Induced by Hemorrhage. VIII. The Inactivation of the Apoenzyme of Amino Acid Oxidase and Lactic Dehydrogenase in Anoxia. Margaret E. Greig....	240
XXIX. The Bone Marrow Procedure for the Assay of Liver Extracts for Anti-Pernicious Anemia Activity. C. M. Young and H. D. Bett ..	248
XXX. A Toxicological and Pharmacological Investigation of Sodium Sec-Butyl Ethyl Barbituric Acid (Butisol Sodium). Charles M. Gruber, Fred W. Ellis and Goldie Freedman	254
XXXI. Clinical Actions of Ethylorsuprarenin. M. L. Tainter, M.D., W. M. Cameron, M.D., L. J. Whitsell, M.D., and M. M. Hartman, M.D..	269
XXXII. The Toxicity and Trypanocidal Activity of p-Sulfonamidophenylarsonic Acid and Certain of Its Derivatives. E. Leong Way and L. K. Chan	278
XXXIII. The Acute Toxicity for Mice of "Mapharsen" and Sodium Sulfathiazole Administered Separately and in Combination. Elizabeth M. Cranston, William G. Clark and Ernest A. Strakoseh	284
XXXIV. Relation of the Intensity of the Morphine Abstinence Syndrome to Dosage. Howard L. Andrews and C. K. Himmelsbach ..	288
XXXV. Inhibition of Nervous Transmission in Synapses and End Plates by Thiamine. K. Unna and E. P. Piek	294
XXXVI. Sulfamerazine (2-Sulfanilamido-4-Methylpyrimidine). III. The Comparative Activity of Sulfamerazine, Sulfadiazine and Sulfapyridine in the Production of Hemolytic Anemia in the Mouse. Albert R. Latven and Arnold D. Welech	301

NUMBER 4, AUGUST, 1944

XXXVII. The Pharmacological Basis for the Rational Use of Atabrine in the Treatment of Malaria. James A. Shannon, David P. Earle, Jr., Bernard B. Brodie, John V. Taggart, Robert W. Berliner and the Resident Staff of the Research Service	307
XXXVIII. Iodine in Blood and Thyroid. VII. An Analytical Procedure for Use in Small Samples: Pharmacological Range of Concentrations. T. S. Sappington, N. Halperin and W. T. Salter ..	331
XXXIX. Changes in Activity of Pulmonary Receptors in Anaesthesia and Their Influence on Respiratory Behavior. D. Whitteridge and E. Bülbring	340

XL	Some Comparative Pharmacological Actions of Beta hydroxy and Methoxy Phenyl n propylamines	Boyd E Graham and George F Cartland	360
XLI	General Analgesic Effects of Procaine	Nolton Bigelow and Irving Harrison	363
XLII	The Oxidation in vitro of Morphine by Rat Liver Slices	Frederick Bernheim and Mary L C Bernheim	374
XLIII	The Digitalis Cat Assay in Relation to Rate of Injection	C I Bliss and M G Allmark	378
XLIV	Streptothricin as a Chemotherapeutic Agent	Harry J Robinson and Dorothy G Smith	390
XLV	Comparative Anticonvulsive Action of 3,5,5 trimethyloxazolidine 2 4 dione (Tridione), Dilantin and Phenobarbital	Guy M Everett and Richard K Richards	402
XLVI	Index		409

THE ABSORPTION AND EXCRETION OF ALLANTOIN IN MAMMALS

E. GORDON YOUNG, HELEN P. WENTWORTH AND W. W. HAWKINS

From The Department of Biochemistry, Dalhousie University, Halifax, Canada

Received for publication November 29, 1943

Allantoin is excreted by most mammals as the final product of purine catabolism. Man excretes uric acid and is a notable exception. Small amounts of allantoin are found however in human urine of the order of about 10-50 mgms. *per diem* (Wiechowski, 1909, 2; Larson, 1931-32; Fosse, Brunel and Thomas, 1931; Paget and Berger, 1938). Aekroyd (1911) has satisfactorily explained this as originating from preformed allantoin in the food. Many studies have been made in the past in an effort to determine the fate of allantoin administered orally or subcutaneously. These are recorded and discussed later. They were done at a time when the metabolic relationship of uric acid and allantoin was being determined.

Due however to the lack of an adequate method of estimating allantoin in blood no studies have been made on the rate of absorption and excretion of allantoin. Over the past few years we have developed in this laboratory suitable techniques for this purpose, (Young and Conway, 1942; Young *et al.*, 1944).

As a result of observations published during the last few years there are indications that in man allantoin may cause a specific physiological effect and serve as a chemo-therapeutic agent. Macalister (1936) has claimed it to be a very effective cell proliferant and leucocytic stimulant. Robinson (1935) attributed the therapeutic action of maggots to the allantoin which they produce and Greenbaum (1940) noted a stimulating effect on leucocyte counts in rabbits. The possible relationship between these two observations occurred to us.

These considerations led us to a more elaborate study of the rate of absorption and excretion of allantoin administered orally, subcutaneously and intravenously to men, dogs and rabbits. These experiments were designed to facilitate an investigation of the leucocytic action of allantoin (Young and Hawkins, 1944, 2).

EXPERIMENTAL. The daily variation in concentration of allantoin in the blood of the dog was first determined for two animals fed Purina dog chow daily at 4.30 p.m. The visual colorimetric method of estimation was employed using 5 ml. of blood (Young *et al.*, 1944). The results are recorded in table 1. They show a fluctuation between 1.2 and 2.3 mgms. per 100 ml. in both animals.

Allantoin administration to dogs. With sodium amytal as anaesthetic dog A was catheterized and 100 ml. of 0.6% allantoin in sterile Ringer's solution were injected intravenously. The rates of disappearance from the blood and excretion in the urine are shown graphically in figure 1. The blood allantoin reached a concentration of 9.5 mgms. 10 minutes after injection, then fell to 2.4 mgm. at 50 minutes and thereafter very slowly for about 5 hours. The concentration of allantoin in the urine rose sharply during the first hour, then fell rapidly for two hours and reached the fasting level in about five hours. If the base line for the

TABLE 1
Allantoin in dog blood

DATE	TIME	ALLANTOIN CONCENTRATION	
		Dog A	Dog B
		<i>mgm.</i>	<i>mgm.</i>
Dec. 3	11.30 a.m.	1.2	2.1
Dec. 10	11.30 a.m.	1.7	1.7
Dec. 12	11.30 a.m.	1.4	1.9
	4.30 p.m.	1.3	
Dec. 16	11.30 a.m.	2.3	1.8
	4.30 p.m.	2.0	1.6
Dec. 17	11.30 a.m.	1.7	1.9
	4.30 p.m.	1.8	2.2
Dec. 18	11.30 a.m.	1.4	1.9
	4.30 p.m.	2.3	2.3
Dec. 19	11.30 a.m.	1.4	1.2
Average		1.7	1.9

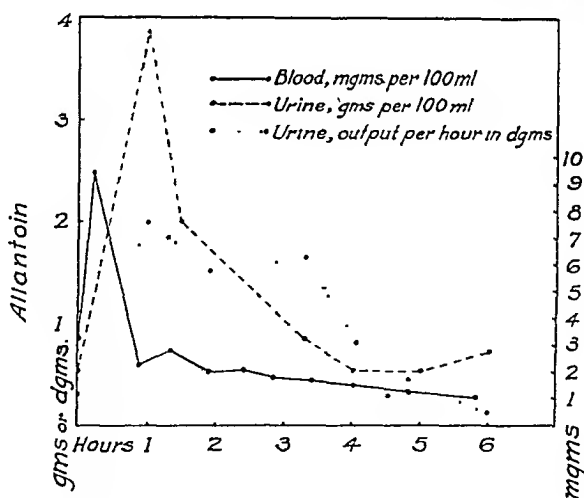


FIG. 1. RATE OF EXCRETION OF ALLANTOIN AFTER INTRAVENOUS INJECTION OF 600 MGm.

excretion of allantoin be taken at 20 mgm. per hour the recovery of added allantoin would be 98% in this experiment. As the urinary concentration is dependent upon available fluid a calculation of the allantoin output per hour was

considered a better criterion of excretion. This was high for the first three hours and fell to normal in 1 to 5 hours. A dose of 600 mgm of allantoin is approximately equivalent to the 24 hour output of this animal, which was thus disposed of in about five hours.

The base line in this and other experiments was determined by keeping the animal in a metabolism cage under routine control and collecting the urine in periods of 12 hours for several days. This permitted the calculation of hourly output for the experimental periods more accurately. During the intravenous administration of allantoin the hourly collection of urine was continued for some time after complete recovery of allantoin to confirm the establishment of this base line.

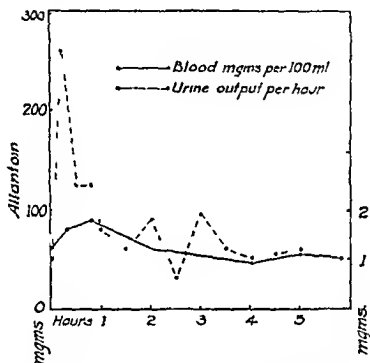


FIG. 2. RATE OF EXCRETION OF ALLANTOIN AFTER INTRAVENOUS INJECTION OF 600 MGm.

Another experiment was performed administering 300 ml of water by stomach tube 30 minutes prior to giving 600 mgm of allantoin as previously. The results are presented graphically in fig. 2. They confirm the previous observations showing somewhat more rapid elimination than in the first experiment, complete in about four hours. Taking the normal rate of excretion at 30 mgm per hour the recovery was 674 mgm or 112 per cent.

Using the same technique it was of interest to us to determine the possible rate of conversion of uric acid to allantoin in these animals. 100 ml of 0.194% uric acid as the lithium salt in sterile Ringer's solution were injected intravenously. 150 ml of water were given by stomach tube 80 minutes prior to the injection. The elimination of both uric acid and allantoin was followed as previously. The results are charted in figs. 3 and 4. The blood allantoin rose to a maximum value in the first hour and fell to normal in the next two while the uric acid returned to the normal level in less than one hour. The uric acid excretion was signifi-

cantly higher only during the first 30 minutes. The allantoin excretion was stimulated for about four hours. Assuming the base line of excretion of allantoin to be 46 mgm. per hour the recovery was 112 mgm., equivalent to 120 mgm. of uric acid or 62%. Assuming the base line for the normal excretion of uric acid to be 2 mgm. per hour, the recovery as uric acid would be 9% of the amount injected or a total of 71% recovered in 3.5 hours. The amount injected was approximately equivalent to this dog's daily output.

The fate of allantoin administered orally has also been studied with these animals. They were kept in metabolism cages and fed 250 gm. of Purina chow daily at 4 p.m. The water intake was also regulated. Creatinine estimations were made on the urine to determine the constancy of the 24 hour specimens. This showed a maximum variation of $\pm 9\%$ for the control period. The allantoin

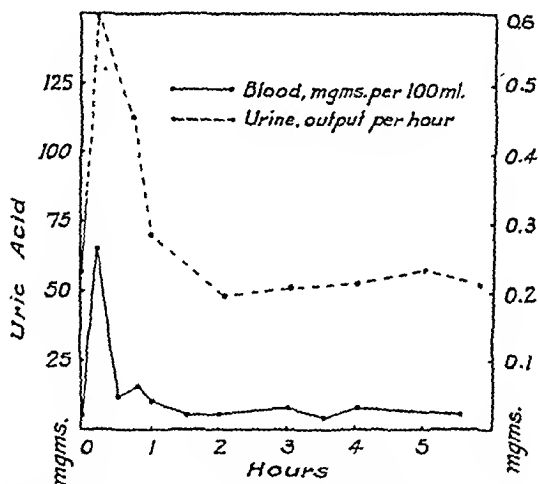


FIG. 3. CONCENTRATION OF URIC ACID IN BLOOD AND URINE AFTER INTRAVENOUS INJECTION OF 194 MGm.

excretion was 600 mgms. ± 75 mgm. or a maximum variation of $\pm 12\%$. Allantoin was given in capsules with the food at dosages of about 400 mgm. In the first three experiments the recoveries were 79, 92 and 67% within 24 hours as shown in table 2. Blood samples taken during the absorptive period never showed abnormal values, fluctuating between 1.4 and 2.0 mgm.

As Purina dog chow contains appreciable amounts of purines a synthetic diet of casein, given at a minimum level, lard and sucrose, supplemented with Harris yeast concentrate, salt mixture, bone ash and halibut liver oil was given in an effort to obtain a more constant base line of allantoin excretion. The level fell from about 600 to 424 mgm. but was not more constant, showing a variation of ± 75 mgm. Experiments with this diet are recorded in table 2 (experiments 4 to 8) showing the recovery in 24 hours. In a few of the experiments listed in

table 2 there was a further small recovery in the second period of 24 hours following the oral dose of allantoin, uncertain numerically because of fluctuation in the base line. The urine was collected in periods of 12 hours or less during the

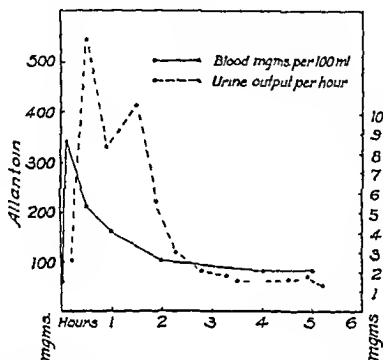


FIG 4 CONCENTRATION OF ALLANTOIN IN BLOOD AND URINE AFTER INTRAVENOUS INJECTION OF 191 MGm OF URIC ACID

TABLE 2

Allantoin excretion after oral administration in dogs

EXPT	AMOUNT GIVEN	FORM OF ADMINISTRATION	RECOVERY IN 24 HOURS	
			Mgm	Per cent
	mgm			
1	392	Capsule	308	79
2	386	Capsule	354	92
3	436	Capsule	303	67
4	571	Capsule	201	35
5	500	Solution—180 ml	375	75
6	517	Capsule	265	76
7	1440	Solution—240 ml	826	57
8	1500	Solution—250 ml	724	48
Average				66

day of administration. In these, frequently more allantoin was recovered in the second 12 hour period.

It is thus apparent that the extent of excretion in the dog after oral administration is lower than after intravenous injection and that the period is prolonged

Allantoin administration to rabbits. Experiments were next performed on the rabbit administering by stomach tube 50 ml. containing 500 mgm. of allantoin in suspension. The animals were fed Purina rabbit chow as basal ration and the daily water intake was kept constant. The urines were diluted to 200 ml. and neutralized before analysis. No additional urinary allantoin was ever found present. The feces were collected and extracted with water. The aqueous extracts were clarified with phosphotungstic acid and basic lead acetate as in the Larson method for allantoin in urine (1931-32). However no allantoin was found to be present.

Allantoin administration to man. Many experiments have been carried out on men using small and large doses administered orally, subcutaneously and intravenously. Due to the presence of uric acid it has been necessary to purify the urine before determining the allantoin by the Young-Conway technique. This was done by applying the first part of the Larson procedure (1931-32) to 5 ml. of urine adding phospho-24 tungstic acid, basic lead acetate and sulfuric acid as prescribed. 5 ml. of this fluid were then diluted to 10 ml. or more as required and neutralized carefully with sodium hydroxide. The Young-Conway technique was then applied to 5 ml. of this material.

Typical results are exemplified in table 3. In oral administration the allantoin was dissolved in water and ingested as one dose of 1 gm. or in one instance in four doses of 1.5 gm. each at two hourly intervals. The urine was collected every two hours or at longer intervals as indicated and until the amount of allantoin present had passed beyond the sensitivity of the method.

For intravenous administration the allantoin was made up as 0.5% solution in Ringer's solution with the aid of gentle heating not exceeding a temperature of 50°. The solutions were then passed through a Mandler filter and injected immediately, intravenously or subcutaneously, with the usual precautions. Doses of 50 to 240 mgm. were used. Only three experiments were tried using subcutaneous injection. At the lowest dosage level the accuracy of estimation of recovery is considerably decreased due to the degree of dilution in the urine. Traces of allantoin were present beyond the stated intervals of collection.

It may therefore be concluded that there is a marked loss or disappearance of allantoin after oral administration. The recovery may be taken as essentially quantitative by the intravenous route, discrepancies being accounted for by the excretion of traces for many hours after the injection. It would appear that subcutaneously the amount recovered is also quantitative although we have only a few experiments at low dosage levels to substantiate this conclusion. At high dosage levels the allantoin continues to be excreted for days after intravenous administration. After a dose of 6 gm. orally however practically all recoverable allantoin had been excreted in two days.

DISCUSSION. Our results are essentially in accord with those published in the literature. These are summarized in table 4. It must be remembered that older methods of estimation were generally rather inaccurate. No previous studies have been made with intravenous administration. There is general

agreement that orally the recovery is low in man and variable in the dog. Our results with the rabbit differ sharply from those of Schaffer and Greenbaum (1940) who claim that the administration of allantoin to this animal serves to stimulate purine metabolism. After subcutaneous injection the recovery by excretion is 75-100% for man, monkey and pig.

TABLE 3
Excretion of allantoin in man

SUBJECT	ADMINISTRATION		INTERVAL (P. URINE COLLECTED)	ALLANTOIN RECOVERY		
	Mode	Amount		Amount	Percentage	Total
R P	oral	1000	2	33.7	3.4	18.6 in 24 hours
			2	48.3	4.8	
			8	73.5	7.4	
			12	30.0	3.0	
E Y	oral	4 × 1000	12	178.5	23.0	33.8 in 72 hours
			12	40.2	6.7	
			24	21.3	3.5	
			24	3.6	0.6	
W H	intravenous	240	24	164.2	68.4	98 in 72 hours
			24	35.8	15.0	
			24	34.5	14.4	
L Y	intravenous	100	4	63.0	63.0	91.5 in 12 hours
			5	22.3	22.3	
			3	9.2	9.2	
T W	intravenous	75	2	25.0	33.0	88.8 in 12 hours
			4	23.6	31.4	
			6	18.3	24.4	
W H	intravenous	50	2	19.2	38.5	71.5 in 6 hours
			4	16.5	33.0	
W F	subcutaneous	50	2	19.2	38.4	81.2 in 6 hours
			4	21.4	42.8	
W H	subcutaneous	50	2	10.3	20.6	73.2 in 24 hours
			4	11.7	23.4	
			18	14.6	29.2	

The explanation for the disappearance of allantoin after oral administration is to be found in bacterial decomposition in the intestine prior to absorption. This has been shown to happen in the feces of the monkey by Givens (1914) and to be brought about by unidentified bacteria from urine by Mendel and Dakin (1909-10).

THE ROLE OF PYROGENS IN THE ALLEGED LEUKOCYTIC RESPONSE TO ALLANTOIN

E. GORDON YOUNG AND W. W. HAWKINS

From the Department of Biochemistry, Dalhousie University, Halifax, Canada

Received for publication November 29, 1943

In 1912 Macalister first suggested the action of allantoin as a cell proliferant and its possible application in the treatment of external and internal ulcers. He reported further observations on allantoin in 1936 and stressed its stimulus to leukocytosis, whether administered orally or parenterally, and its use in lobar pneumonia using a dose as low as 10 mgm. The position of allantoin in therapeutics was reviewed by the Council on Pharmacy and Chemistry of the American Medical Association in 1938 and judged not established on the evidence then available. Since then Greenbaum (1940) has claimed to have produced a neutrophilic leukocytosis in rabbits and dogs after intramuscular, intravenous and oral administration. The possible relationship between the leukocytosis and the function of allantoin in wound healing as in maggot therapy (Robinson, 1935) caused us to investigate more thoroughly the relationship between allantoin and leukocytosis especially in man.

For the reason that our results were finally interpreted as negative in man, dog and rabbit, they are presented below only as a few examples of a large group of experiments extending over a period of two years.

EXPERIMENTAL. Three different specimens of allantoin made by Merck & Company, Eastman Kodak Company and British Drug Houses have been used at different times. These were recrystallized from warm water for some of the experiments. Due to preliminary charring, melting point determinations tend to vary considerably and end in decomposition. Typical results were 227°, 230°-232°, 234°. Heilbron (1934) lists 235°-236°C.

Solutions were always freshly prepared on the day of administration a short time before use, warming to a temperature of 50°-60°C. They were sterilized by passage through a fine Mandler or Berkefeld filter. The medium was Ringer's solution or physiological saline.

Red and white cell counts were made with a good Zeiss hemacytometer on peripheral blood. In every case at least two counts were made. Differential smears were colored with Wright's stain and usually 200 cells were counted.

It has been our practice to study the normal variations in the counts of the experimental subject for several days and at different times of the day prior to administering allantoin. That this is a necessary procedure before the conclusion can be drawn of a leukocytosis may be judged by the variations listed in table 1 of eleven normal healthy males from 20 to 45 years of age who have served as experimental subjects.

The only regularity in fluctuation that appeared in our counts was that the highest count which the individual showed appeared with few exceptions about

the middle of the afternoon and the lowest count in the early morning. Exercise and food ingestion did not appear to influence the white cell count or any one type of cell. This is in agreement with the observations of Shaw (1927) and others (Sturgis and Bethell, 1943).

Oral administration in man Macalister (1936) and his colleagues claimed to have induced a leukocytosis with repeated oral doses as low as 60-130 mgm. Five individuals have ingested single multiple or doses of allantoin ranging from 0.26-7.35 gm. either in capsules or in solution. Taking into account the normal fluctuations of each individual no increase in the number of leukocytes or of any one type of cell could be detected. Most of the allantoin ingested is decomposed by bacteria in the intestine (Young, Wentworth and Hawkins, 1944). One typical experiment at a high dosage level is shown in table 2 in which 34% of the allantoin was recovered in the urine. All figures recorded are within the normal

TABLE 1
Normal fluctuations in leukocyte counts of healthy males

SUBJECT	WHITE BLOOD CELLS	DIFFERENCE	NEUTROPHILS	DIFFERENCE
	per c mm		%	
W. H.	6200- 9,200	3000	50-70	20
E Y	5200-10,100	4900	49-65	16
R P.	4300- 6,600	2300	33-53	25
W F	3800- 6,500	2700	48-57	0
F R	4700- 7,100	2400	50-59	9
G H.	5600- 8,900	3500	46-61	15
T W.	5500- 6,700	1200	52-70	18
C. W	5000- 6,900	1900	48-61	13
G M.	4200- 6,600	2400	47-63	16
B C	5800- 8,700	2900	53-71	18
M M	6000- 8,200	2200	46-59	13
Average		2673		16

range for this individual. No subjective symptoms were noted in any of these experiments. Table 3 records an experiment with multiple doses of about one grain in repetition of the procedure suggested by Macalister (1936). No leukocytosis was evident.

Intravenous administration in man. No observations on intravenous injection of allantoin in man have been recorded in the literature to our knowledge. Macalister (1936) has claimed that a subcutaneous dose of 10 mgm. to patients with pneumonia raised the leukocyte count in some cases but no case records are submitted and few details are given. We have carried out a large series of experiments on eleven male adults with doses of 50-240 mgm. dissolved in physiological saline or in Ringer's solution at a concentration of about 0.5% allantoin. The response was sometimes doubtful at 50 mgm, in 10 ml., but usually detectable at 75 mgm. in 15 ml. There was a definite leukocytosis with a specific neutrophilia occurring in about 3-4 hours and lasting for 5-6 hours or longer. Occasionally a

leukopenia was detectable about an hour after the injection. In no case was there any significant alteration in the red blood cell counts. A typical result is shown in table 4.

The response to the largest dose given intravenously, 240 mgm. in 60 ml. of Ringer's solution, is tabulated in table 5. The leukocytosis appeared between the third and sixth hour and remained for many hours. The neutrophilia was

TABLE 2
Effect of oral administration in man

Subject: E. G. Y. Normal W.B.C. variation: 5,200-10,000. Percentage variation in neutrophils: 49-65. Dosage: 6 gm. as 4 doses of 1.5 gm. in 400 ml. water.

TIME	W B C. PER MM	DIFFERENTIAL COUNT					REMARKS
		M	L	N	E	B	
10.30 a.m.	7,900	2.0	39.5	56.0	2.0	0.5	1st dose of 1.5 gm.
12.45 p.m.	6,700	0.3	45.3	52.0	1.7	0.7	2nd dose of 1.5 gm.
3.00 p.m.	5,200	0.0	34.5	65.0	0.5	0.0	3rd dose of 1.5 gm.
6.00 p.m.	7,800	0.3	44.0	54.3	1.3	0.0	4th dose of 1.5 gm.
8.00 p.m.	8,600	0.5	47.5	49.5	1.5	1.0	
10.15 p.m.	6,600	0.0	47.0	52.5	0.5	0.0	

TABLE 3
Effect of oral administration in man

Subject: R. P. Normal W.B.C. variation: 4,300-6,600. Percentage variation in neutrophils: 33-58. Dosage: 260 mgm. as 4 doses of 65 mgm. in 40 ml. water.

TIME	W B C	DIFFERENTIAL COUNT					REMARKS
		M	L	N	E	B	
8.45 a.m.	5,500	0.5	43.5	55.0	0.5	0.5	1st dose
10.30 a.m.	6,300	0.7	44.3	54.3	0.7	0.0	
10.45 a.m.	5,200	0.0	45.0	54.0	0.5	0.5	2nd dose
12.45 a.m.	5,200	1.0	42.0	56.0	0.0	1.0	3rd dose
2.00 p.m.	6,000	0.5	50.5	48.5	0.5	0.0	
2.45 p.m.		0.5	55.5	44.0	0.0	0.0	4th dose
3.30 p.m.	5,400	1.0	48.5	49.5	0.5	0.5	
4.15 p.m.	5,500	1.0	50.5	47.0	1.5	0.0	
7.00 p.m.	6,300	1.0	46.5	49.5	2.0	1.0	

definitely present throughout the second day. No appreciable leukopenia was shown by this individual. Of the dose administered 98% was estimated in the urine, 69% on the first day, 15% on the second and 14% on the third.

Subcutaneous administration in man. Several subcutaneous injections were carried out with doses of 25-50 mgm. dissolved in Ringer's solution. The initial results were very similar to those obtained after intravenous administration.

A leukocytosis with a neutrophilia was apparent in about 4-5 hours lasting for only a few hours at this dose level

In attempting to determine the minimum dose of allantoin which would produce a definite leukocytosis the number of subjects was extended with the

TABLE 4

Effect of intravenous administration in man

Subject T W Normal W B C variation 5 500-6 700 Percentage variation in neutrophils 52.70 Dosage 75 mgm in 15 ml Ringer's solution

TIME	W.B.C	DIFFERENTIAL COUNT					REMARKS
		M	L	N	E	B	
11 30 a m	6 400	2.3	36.6	61.0	0.1	0.0	Allantoin injected
1 00 p m	3 900	0.0	21.0	77.5	1.5	0.0	
2 30 p m	13 300	0.2	7.5	92.3	0.0	0.0	
4 30 p m	10 400	0.5	9.0	90.0	0.5	0.0	
5 30 p m	7 400	3.0	15.0	81.5	0.5	0.0	
7 45 p m	7 300	0.5	23.5	75.0	1.0	0.0	
8 45 p m	6 700	2.5	22.0	74.0	0.5	1.0	

TABLE 5

Effect of intravenous administration in man

Subject W H Normal W B C variation 6 200-9 200 Percentage variation in neutrophils 50-70 Dosage 210 mgm in 60 ml Ringer's solution

TIME	W B C	DIFFERENTIAL COUNT					REMARKS
		M	L	N	E	B	
1st day							Allantoin injected
12 45 p m	7 200	2.0	35.5	59.5	2.5	0.5	
1 45 p m	6 200	0.0	33.0	66.0	0.5	0.5	
3 45 p m	7 600	0.0	35.5	63.5	1.5	0.0	
6 15 p m	15 500	0.5	9.7	89.3	0.5	0.0	
6 45 p m	15 800	0.5	13.0	85.5	1.0	0.0	
11 00 p m	14 900	0.5	22.5	76.5	0.5	0.0	
2nd day							
10 30 a m	8 700	0.7	17.0	81.0	1.3	0.0	
1 00 p m	9 100	1.0	15.0	82.5	1.0	0.5	
8 30 p m	9 600	0.0	16.0	81.5	2.0	0.5	
3rd day							
11 15 a m	6 100	2.0	29.0	68.5	0.5	0.0	

appearance of symptoms of shock in some individuals. Several subjects complained of headache, nausea and vomiting. They ran a hyperpyrexia of 99-104° F which coincided with the leukocytosis and neutrophilia.

Control experiments were then done administering 15 ml of Ringer's solution

sterilized by passage through the same filters as used previously to subjects who had previously not experienced any symptoms. In one instance, shown in table 6, the patient experienced no physical discomfort but a definite leukocytosis with a neutrophilia was apparent and there was also a slight hyperpyrexia. In another experiment using 15 ml. of the same Ringer's solution some days later a marked reaction was experienced with a leukopenia of 2,600 in one hour and a leukocytosis of 15,200 in six hours with a neutrophilia at 96% of the white cells. The oral temperature reached 103°F. in four hours. There was a marked shift to the left in the blood picture. Such findings strongly suggested the presence of pyrogenic substances in the solvent (Seibert, 1923; Seibert and Mendel, 1923; Banks, 1934; Co Tui *et al.*, 1937; Co Tui and Schrift, 1942). Using doubly or triply distilled water from all-glass stills and sterilizing by autoclave, isotonic saline solutions produced no effect on either the white cell counts or the percentage of neutrophils. There were no subjective symptoms.

TABLE 6

Control experiment with Ringer's solution

Subject: W. H. Normal W.B.C. variation: 6,200-9,200. Percentage variation in neutrophils: 50-70. Dosage: 15 ml. Ringer's solution intravenously.

TIME	W B C.	DIFFERENTIAL COUNT					REMARKS
		M	L	N	E	B	
6.00 p.m.	6,700	7.0	37.0	56.0	0.0	0.0	Ringer solution
8.30 p.m.	11,400	3.0	16.5	79.0	0.5	1.0	
9.30 p.m.	13,700	3.5	12.5	83.0	1.0	0.0	Temperature 99.4°F.
11.00 p.m.	10,700	2.5	23.0	74.0	0.5	0.0	
12.05 a.m.	9,200	6.0	19.0	74.0	0.0	1.0	Temperature 99.4°F
1.00 a.m.	9,500	2.5	25.5	71.5	0.5	0.0	
8.30 a.m.	8,400	3.5	19.5	76.0	1.0	0.0	Temperature 98.6°F
9.00 a.m.	5,400	3.0	39.0	57.0	1.0	0.0	

The allantoin experiments were then repeated using 75 mgm. in fresh saline made up in doubly distilled water sterilized by passage through a Berkefeld filter as before. There was no leukocytosis or neutrophilia in four experiments. A fifth experiment was carried out using 200 mgm. of a recrystallized specimen in 40 ml. saline solution. The results are shown in table 7 and they are definitely negative despite the large dose administered.

Allantoin administration to dogs and rabbits. In the course of this investigation the effect of allantoin has been tested on rabbits and dogs. Careful and prolonged observations have been made on three rabbits to determine their normal variation in w.b.c. and differential counts. The white cell count showed a tendency to rapid and wide fluctuations with an increase of possibly 100% over a period of a few hours. The proportionality between neutrophils and lymphocytes also tended to change rapidly and unpredictably. This is notable in counts recorded by Cheng (1930) and also in a recent paper by Chapman (1942). Selecting one animal that had shown the most constant counts, viz., 5,700-9,600, avg. 7,700 w.b.c., and 37-71% neutrophils, avg. 52%, an experiment was carried out administering 500 mgm. allantoin orally in five gelatin capsules. The w.b.c.

count rose to 12,300 in four hours and the neutrophilia to 75%. These results were not convincing because counts made on the second and third days showed wide fluctuations and a count of 13,800 with 39% neutrophils occurred on the third day. Some experiments were, however, suggestive of a definite leukocytosis at 18,300 with a neutrophilia.

The dog was a better experimental animal because of more constant counts. In one experiment 1.5 gm. of allantoin in solution were given by stomach tube without any significant rise in the w.b.c. count. In three experiments doses of 100 mgm. and in one a dose of 270 mgm. given either intravenously or intramuscularly were accompanied by increases of about 25% or no change at all. These increases we do not consider statistically significant.

TABLE 7

Effect of allantoin in pyrogen-free saline solution

Subject: W. H. Normal W.B.C. variation: 6,200-9,200. Percentage variation in neutrophils: 50-70. Dosage: 200 mgm. in 40 ml. saline intravenously.

TIME	W B C	DIFFERENTIAL COUNT					REMARKS
		M	L	N	E	B	
11.30 a.m.....	5,200	5.0	30.5	62.0	2.0	0.5	Allantoin injected
12.30 p.m.....	6,200	5.5	23.0	70.0	1.0	0.5	
1.30 p.m.....	6,900	8.5	29.0	61.0	0.5	1.0	
2.30 p.m.....	7,000	5.5	31.5	61.0	1.0	1.0	
3.30 p.m.....	7,100	8.0	38.5	52.5	0.0	1.0	
4.30 p.m.....	7,100	5.0	29.5	64.0	1.5	0.0	
6.15 p.m.....	7,300	4.0	33.5	60.0	2.0	0.5	
11.30 p.m.....	6,400	7.5	40.0	50.5	2.0	0.0	

Discussion. The evidence for a leukocytosis from allantoin was adduced for man by Macalister (1936) and for rabbits and dogs by Greenbaum (1940). The former used doses of 60-130 mgm. orally and 10 mgm. parenterally and neglected to take into consideration normal fluctuations in w.b.c. counts accepting as significant increases of 5 to 15% in polymorphonuclear cells and 25 to 47% in total counts. Our observations would permit such fluctuations in normal individuals as shown in table 1. The doses used by us have duplicated and far exceeded those used by Macalister without convincing increases in counts in the absence of pyrogenic substances.

Greenbaum has claimed that 0.5 gm. given in suspension by stomach tube to rabbits produced a definite leukocytosis in 1-2 hours. Intramuscular injections of 20 mgm. to three animals were found to be more effective. Intravenous injections of 20-40 mgm. in single or repeated doses were less effective and a critical appraisal of the counts would judge them unchanged in our opinion. The effect on dogs in Greenbaum's experiments were less marked than on rabbits. We do not consider that the counts after administration of allantoin recorded by Greenbaum or Macalister are convincing because the normal fluctuations were not adequately taken into consideration.

We do not therefore believe that allantoin can be classed with nucleotides, adenine or guanine as a leukocytic agent. Furthermore it would be surprising to

The averages of these tests, summarized in table 1, show that at pH 6.0, sulfamethazine and its N⁴-acetyl derivative were considerably more soluble in buffer or urine than sulfamerazine and its acetyl derivative, which in turn were more soluble than the respective sulfadiazines. As the pH increased toward 7.0, these solubility differences diminished and at pH 7.6 or 8.0 were essentially absent. It should be noted that in nearly all cases the acetyl derivatives were somewhat more soluble than the free sulfonamides. It should also be pointed out that the pH of solutions of the acetyl derivatives which were initially above 7.0 became more acid during the period of incubation. This change may have resulted from hydrolysis of the acetylated drugs, since free sulfonamide was detected in the solutions with pH greater than 7.0.

TABLE 1

Solubilities of sulfadiazine (SD), sulfamerazine (SMD), sulfamethazine (SMMD), and their N⁴-acetyl derivatives in phosphate buffer and human urine

INITIAL pH OF FLUID	MG. % SULFONAMIDE IN SOLUTION					
	SO	SMO	SMMD	AcSD	AcSMO	AcSMMD
Solubility in phosphate buffer						
6.0	16.8	36.5	67.2	27.6	37.8	70.4
6.4	24.6	43.9	68.6	48.1	62.6	80.0
6.8	41.6	61.4	77.0	80.1	100.5	95.0
7.2	78.5	98.4	96.0	120.9	123.4	115.0
7.6	130.0	148.7	128.3	212.0	194.0	186.5
8.0	177.5	199.0	165.5	230.5	227.0	233.0
Solubility in urine						
6.0	16.8	38.4	69.2	30.4	39.0	67.0
6.4	30.9	48.4	75.2	49.5	78.0	86.4
6.8	41.6	62.8	83.3	92.0	108.0	90.7
7.2	81.8	86.3	99.7	110.0	113.4	114.0
7.6	138.7	158.3	144.5	210.0	204.8	176.2
8.0	186.5	208.5	179.3	229.1	227.2	216.0

The above data are similar to those reported by Gilligan and Plummer (9), who used essentially the same procedures in determining solubility. The only significant difference in the results is that Gilligan found sulfadiazine to be much more soluble at pH 7.8 than sulfamerazine and sulfamethazine, whereas in the current experiments the three drugs had essentially the same solubility at this pH. The explanation for this difference is not apparent. Both the present data and those of Gilligan differ from the data reported for sulfadiazine by Jensen and Fox (10), for sulfamerazine by Weleh and coworkers (11), and for sulfamethazine by Macartney (12) and Rose (6). These observers reported much greater solubilities for all derivatives at alkaline pH than were observed here, although their data at acid pH are not significantly different. It is generally recognized that the technique of determining solubility of the sulfonamides has a

marked effect on the absolute results, furthermore, it is an easy matter to obtain supersaturated solutions. These factors may well explain the divergent results noted above, since the procedures employed by these workers were quite different from those used in the present study.

Assuming that the renal toxicity of a sulfonamide is to a great extent inversely proportional to its solubility, the question may be raised whether the differences in solubility of sulfamethazine, sulfamerazine and sulfadiazine at acid pH are great enough to suggest a significant difference in renal toxicity. It is true that the absolute differences in the solubility of these drugs are small, the relative differences are considerable, however, sulfadiazine at pH 6.0 being only one half as soluble as sulfamerazine and one fourth as soluble as sulfamethazine. That these differences in solubility are important in determining renal toxicity will be shown in the rat and dog experiments described in a later section of this study.

B Absorption and excretion. Absorption studies on sulfadiazine, sulfamerazine and sulfamethazine were carried out in mice, rats and monkeys. With minor exceptions the technique of each experiment was the same. All animals received the sulfonamides by stomach tube. In the experiments with white mice (18 to 20 grams), groups of 3 animals were sacrificed 1, 2, 4 or 8 hours after receiving unit quantities of the above drugs, suspended in 0.2 cc of water. The free sulfonamide concentration in the blood of each animal was determined and the results averaged. In the experiments with white rats (130-150 grams), groups of 6 animals received the desired quantity of drug suspended in 1 cc of water. Heart blood samples were obtained from each of these animals 1, 2, 4 and 8 hours after treatment, these samples were also analyzed individually for free sulfonamide content and the results averaged. In the experiments with monkeys, the animals received the drugs suspended in 50 cc of water and were bled from the cephalic vein at the intervals indicated in table 2. The monkeys were fasted throughout the experimental period, the food and water supplies of the mice and rats were not restricted.

The concentrations of sulfadiazine, sulfamerazine and sulfamethazine in the blood following oral administration of these drugs are shown in table 2. The data in this table show, first, a significant difference in the time at which peak blood levels of the three drugs were attained. In the work with mice and rats, maximum blood levels of sulfamerazine and sulfamethazine were attained more rapidly than were those of sulfadiazine. In the experiments with rats, peak levels of sulfamethazine were found at earlier periods than those of sulfamerazine. These relationships were not apparent in the experiments with monkeys. See only, the data show distinct differences in the rates at which the different sulfonamides disappeared from the blood. Thus 8 hours after the drugs had been administered, the concentrations of sulfamerazine were distinctly higher than those of sulfadiazine and sulfamethazine. This phenomenon was most striking in the experiments with monkeys. In connection with the chronic experiments to be described later, it should also be noted that in the monkeys the 8-hour levels of sulfadiazine were distinctly lower than those of sulfamethazine.

The excretion of the three sulfonamides was studied indirectly, by determining

their elimination from the blood following intravenous administration. Admittedly the validity of this procedure would depend on similar distribution of the

TABLE 2

Concentrations of sulfadiazine, sulfamerazine and sulfamethazine in blood following oral administration of single doses

DOSE PER KG. BODY WEIGHT	DRUG*	MG. % FREE SULFONAMIDE IN BLOOD				
		Hours after ingestion of drug				
		1	2	3	4	8
Experiments with mice†						
grams						
0.25	SD	5.3	6.3		4.0	1.7
	SMD	6.7	6.3		4.6	2.3
	SMMD	5.3	4.2		3.6	1.6
0.5	SD	8.9	10.8		9.1	2.6
	SMD	11.0	9.3		7.3	4.1
	SMMD	11.5	8.5		6.9	3.4
Experiments with rats‡						
0.125	SD	6.6	10.2		11.9	8.4
	SMD	11.9	14.4		14.7	10.5
	SMMD	12.6	12.5		9.8	6.8
0.25	SD	11.4	18.5		20.9	16.6
	SMD	22.7	25.5		25.5	19.7
	SMMD	22.5	21.1		17.7	15.3
Experiments with monkeys§						
0.1	SD	2.7	3.4	5.1	6.0	2.7
	SMD	5.0	8.5	11.0	11.5	7.7
	SMMD	4.6	7.9	12.5	12.7	6.1
0.4	SD	4.5	7.1	7.3	6.2	4.2
	SMD	10.0	17.3	21.7	19.0	14.4
	SMMD	4.4	12.7	16.4	13.5	8.5

* Sulfadiazine = SD; sulfamerazine = SMD; sulfamethazine = SMMD.

† Each figure represents the average sulfonamide concentration in the blood of 3 mice sacrificed 1, 2, 4 or 8 hours after receiving the indicated dosage of drug.

‡ Each figure represents the average sulfonamide concentration in the blood of 6 rats. These animals were bled 1, 2, 4 and 8 hours after receiving the indicated dosage of drug.

§ Each figure represents the sulfonamide concentration in the blood of one animal, bled at the intervals indicated in the table.

three drugs in the tissues. Whether this is the case has not yet been determined.

Rats and monkeys were used in these experiments. Three rats were employed

for each dosage. The required quantity of the sodium sulfonamide was injected into the tail vein. Heart blood samples were obtained at 1, 2, 4 and 8 hour intervals and analyzed as described above. The monkeys received the requisite amount of the sodium sulfonamide via the cephalic vein.

The concentrations of sulfadiazine, sulfamerazine and sulfamethazine in the blood following intravenous administration of these drugs are shown in table 3. The data indicate that sulfamerazine is removed from the blood stream more

TABLE 3

Concentrations of sulfadiazine, sulfamerazine and sulfamethazine in blood following intravenous injection of single doses

DOSE PER KG BODY WEIGHT	DRUG*	MCN % FREE SULFONAMIDE IN BLOOD			
		Hours after injection of drug			
		1	2	4	8
Experiments with rats†					
0.05 grams	SD	10.7	9.5	8.3	6.5
	SMD	12.1	10.5	9.2	7.3
	SMMD	9.5	8.6	6.6	4.5
0.2	SD	32.4	27.7	22.5	14.1
	SMD	31.2	27.7	22.1	17.3
	SMMD	26.6	23.1	18.5	13.2
Experiments with monkeys‡					
0.025	SD	4.9	4.2	0.0	0.0
	SMD	7.1	6.0	3.4	0.9
	SMMD	4.6	4.2	0.8	0.0
0.1	SD	14.9	9.0	7.0	2.3
	SMD	19.0	17.3	13.4	5.6
	SMMD	16.5	14.3	8.5	3.4

* Sulfadiazine = SD, sulfamerazine = SMD, sulfamethazine = SMMD

† Each figure represents the average sulfonamide concentration in the blood of 3 rats. These animals were bled 1, 2, 4 and 8 hours after receiving the indicated dosage of drug.

‡ Each figure represents the sulfonamide concentration in the blood of one animal, bled at the intervals indicated in the table.

slowly than sulfamethazine or sulfadiazine. The present experimental data permit no definite conclusions as to the relative rates of removal of the two latter drugs.

The observations recorded in tables 2 and 3 suggest that sulfamerazine and sulfamethazine are absorbed more rapidly than sulfadiazine, also that sulfamerazine is excreted more slowly than the other two drugs. These observations are in fair agreement with the results of the more complete study of Welch *et al* (11), with the preliminary report of Goodwin and Finland (14) on the absorption of

these drugs, and with the experiments of Welch (11) and of Marshall and Shannon (13) on excretion of the compounds. Attention should be called to the fact that the comparatively small differences in absorption and excretion indicated in the present single dose experiments may assume considerable significance during continued administration of the drugs as in ordinary therapeutic usage or in chronic toxicity studies.

C. Acute toxicity. The acute toxicity of sulfadiazine, sulfamerazine and sulfamethazine was determined in white mice (13 to 15 grams weight). The toxicity of the free drugs was determined orally, subcutaneously and intraperitoneally; that of the *N*-acetyl derivatives was determined intraperitoneally only. Regardless of the route of administration, the dose of sodium sulfonamide containing the calculated amount of the acid drug was administered in a volume of 0.5 cc. All deaths occurring within 48 hours of treatment were considered acute deaths and were included in the data from which LD_{50} 's were calculated.

In order to assess the acute toxicity of the drugs more completely, the blood levels associated with LD_{50} 's were also determined. Groups of 6 mice were sacrificed 1, 2 or 4 hours after treatment with the requisite amounts of the various drugs. Heart blood samples were obtained and analyzed for free or total sulfonamide content, depending upon whether the free or acetylated drug had been administered.

The results of these experiments are summarized in table 4. According to both LD_{50} data and those on the peak sulfonamide concentrations in the blood, sulfamethazine had greater subcutaneous and intraperitoneal toxicity than sulfamerazine, which in turn was slightly more toxic than sulfadiazine. The oral toxicity of the drugs did not fall in this order. According to the LD_{50} data, sulfadiazine and sulfamethazine reacted similarly but had considerably greater oral toxicity than sulfamerazine. According to the blood level data, sulfadiazine was more toxic than either sulfamerazine or sulfamethazine, which were of equal toxicity.

The experiments with the acetyl derivatives are of particular interest. According to both LD_{50} and blood level data, acetyl sulfamethazine was considerably less toxic than acetyl sulfadiazine or acetyl sulfamerazine. In comparing the toxicity of the acetylated and non-acetylated drugs, it is of interest to note that, blood level for blood level, acetyl and free sulfamethazine had essentially the same toxicity, whereas acetyl sulfadiazine and acetyl sulfamerazine were nearly twice as toxic as the respective free drugs.

It should also be pointed out that animals receiving lethal doses of sulfamerazine, sulfamethazine or their acetyl derivatives died within 6 hours of treatment. This was in striking contrast to the results with sulfadiazine and acetyl sulfadiazine. Mice receiving these drugs died 18 to 36 hours after treatment. This delayed death among mice receiving sulfadiazine has been reported previously (15-16).

D. Chronic toxicity. The chronic toxicity of sulfadiazine, sulfamerazine and sulfamethazine has been compared in rats, dogs and monkeys. Particular attention was paid to the effects of the drugs on body growth and the development

TABLE 4

The acute toxicities of sulfadiazine, sulfamerazine and sulfamethazine and their N⁴ acetyl derivatives

DOSE PER ACM BODY WEIGHT	SULFADIAZINE			SULFAMERAZINE			SULFAMETHAZINE		
	No deaths per no mice treated	L D 50 approx	Concen- tration in blood*	No deaths per no mice treated	L D 50 approx	Concen- tration in blood*	No deaths per no mice treated	L D 50 approx	Concen- tration in blood*
<i>Free sulfonamides</i>									
<i>Oral toxicity</i>									
grams			mgm %			mgm %			mgm %
5.0	30/30			28/30			30/30		
4.0	76/80			40/55	3.3	148	50/60		
3.0	63/75			26/55			52/60		
2.0	45/75	1.8	102	4/55			39/60	1.9	166
1.0	7/50			0/25			12/55		
<i>Subcutaneous toxicity</i>									
2.0	25/35			36/40			15/15		
1.75	17/30	1.6	180	23/30	1.6	164	15/15		
1.5	16/40			15/45			24/25		
1.25	6/30			7/30			17/25	1.1	123
1.0	0/30			0/30			8/25		
0.75							2/25		
<i>Intraperitoneal toxicity</i>									
2.0	34/35			38/40			15/15		
1.75	24/30	1.6	206	27/35					
1.5	19/40			22/40	1.4	161	25/25		
1.25	5/30			12/30			25/25		
1.0	0/25			2/30			22/25	0.9	120
0.75							1/25		
<i>N⁴ acetyl sulfonamides</i>									
<i>Intraperitoneal toxicity</i>									
2.0							40/40		
1.75							29/30		
1.5							25/35	1.3	105
1.25	35/35			62/65			24/60		
1.0	39/40			48/65			1/40		
0.75	42/65	0.6	70	37/65	0.7	66			
0.5	30/65			12/65					
0.25	0/30			0/30					

* The concentration reported here is the average peak concentration. Peak concentrations of sulfamerazine and sulfamethazine were attained 1 hour after administration of these drugs; the peak concentration of sulfadiazine was attained 2 hours after treatment.

of urinary tract pathology. In addition, in the monkeys and dogs a complete histopathological study was made.

pelvis, the pathology was limited to a moderate dilatation of the collecting tubules in scattered areas of the medulla, the tubular epithelium was entirely normal, however, and there was no cellular reaction (In table 7, such kidneys

TABLE 7

The renal toxicity of sulfadiazine, sulfamerazine and sulfamethazine

MG% PER CENT SULFONAMIDE IN BLOOD*	PER CENT OF RATS WITH URINARY TRACT CONCRETIONS	RESULT OF MICROSCOPIC EXAMINATION			
		Per cent of kidneys			
		Normal†	Showing slight pathology‡	Showing moderate pathology§	Showing severe pathology
Rats on diets containing sulfadiazine					
10- 20	0	81	15	0	4
21- 40	3	21	16	26	37
41- 60	5	1	9	13	77
61- 80	8	0	0	17	83
Rats on diets containing sulfamerazine					
10- 20	0	100	0	0	0
21- 40	0	97	3	0	0
41- 60	32	66	29	5	0
61- 80	65	48	43	9	0
81-100	89	28	28	44	0
Rats on diets containing sulfamethazine					
10-20	0	100	0	0	0
21-40	0	100	0	0	0
41-60	0	100	0	0	0
61-80	0	100	0	0	0
Rats on control diets					
0	0	100	0	0	0

* Average of levels obtained at 1 and 2 weeks. Number of rats in each blood level group the same as in table 6.

† Figure 1 illustrates the microscopic structure of the normal kidney.

‡ Figure 2 illustrates the microscopic structure characteristic of the kidney with "slight pathology."

§ Figure 3 illustrates the microscopic structure characteristic of the kidney with "moderate pathology."

|| Figure 4 illustrates the microscopic structure characteristic of the kidney with "severe pathology."

have been classified as showing *slight pathology*, cf. figure 2.) More severe lesions were found in those rats in which the concretions blocked free flow of urine. In these cases, there was greater and more widespread dilatation of the collecting

tubules. In many cases the entire pyramid was involved and in some cases the cortex and papilla also. The tubular epithelium was entirely normal but the lumens of scattered tubules contained small numbers of polymorphonuclear leucocytes. Convoluted tubules and renal corpuscles were not affected. (In table 7, such kidneys have been classified as showing *moderate pathology* of figure 3.)

Although sulfadiazine concentrations occurred less frequently, this sulfonamide had much greater toxicity for the kidney than sulfamerazine. Microscopic evidence of pathology was found in 19% of the kidneys from rats having sulfadiazine blood levels of 10 to 20 mgm % and in 79% of the kidneys from animals having levels of 21 to 40 mgm %. In contrast to this only 3% of the kidneys of rats having sulfamerazine levels of 21 to 40 mgm % showed pathological changes. In a comparatively few instances the lesions in the sulfadiazine animals were similar to those described as *slight* or *moderate* in the sulfamerazine group. In the majority of cases however, the pathological changes were considerably more severe. In these cases there was enormous dilatation of the collecting tubules throughout the kidney. The cells of many of these tubules showed degenerative changes and the lumens were filled with polymorphonuclear leucocytes, cellular debris and at times crystalline drug. Masses of leucocytes had accumulated between the tubules. Often degenerated tubules had been replaced by fibrous tissue. The renal corpuscles were normal, as were most of the convoluted tubules; in a few cases, however, the convoluted tubules were compressed, probably as a result of extreme dilatation of the collecting tubules. (In table 7 such kidneys have been classified as showing *severe pathology* of figure 4.)

The urinary tract or renal toxicities of sulfamethiazine, sulfamerazine and sulfadiazine seem to reflect the solubilities of these drugs at an acid pH—sulfamethiazine the most soluble drug having the least toxicity, and sulfadiazine the least soluble drug having the most toxicity. Since rat urine has an acid pH (4.5 to 6.0 on a diet of Purina Dog Chow Meal) there is a temptation to ascribe the renal toxicity differences to the solubilities of the drugs. Other factors may also be responsible at least in the case of sulfamerazine and sulfadiazine. Marshall (13) and Shinnon (13) have shown significant differences in the renal clearance of these two compounds, sulfamerazine clearance being only one half that of sulfadiazine. These differences in clearance coupled with the solubility differences may be responsible for the greater toxicity of sulfadiazine. Although at present there is no evidence to support this viewpoint, the possibility must also be considered that the three sulfonamides or their acetyl derivatives possess different degrees of toxicity for the tubular epithelium.

The tendency of sulfamerazine to deposit in the renal pelvis whereas sulfadiazine deposits in the collecting tubules probably again reflects the differences in solubility and renal clearance. Sulfadiazine, being the less soluble of these drugs and eliminated in higher concentration, tends to deposit higher up in the renal system.

2. *Experiments with dogs.* Eighteen mongrel dogs were used in these experiments;⁴ groups of 5 animals received sulfadiazine, sulfamerazine or sulfamethazine, the 3 remaining

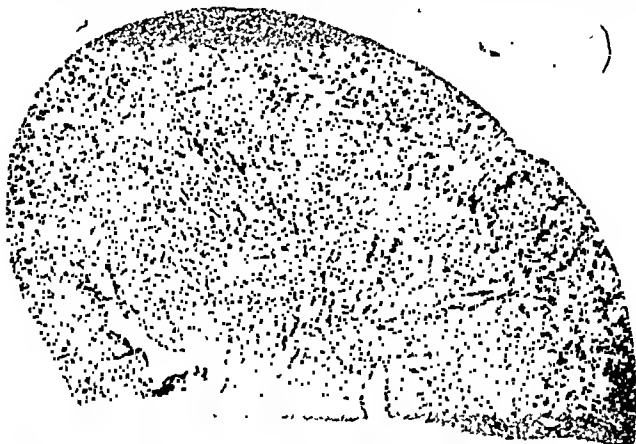


FIG. 1. Plate A

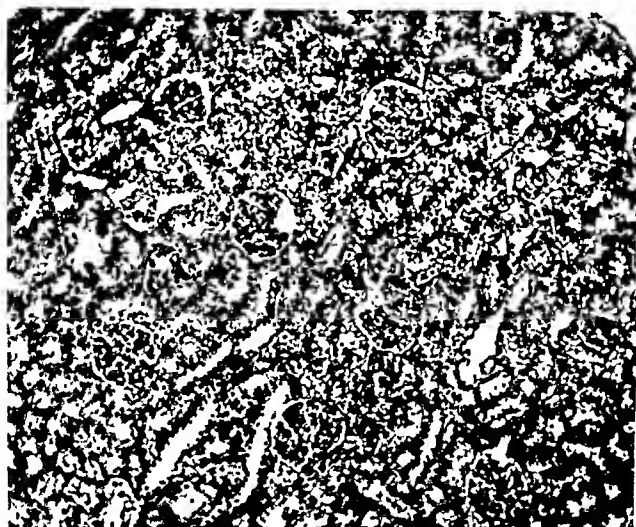


FIG. 1. Plate B

dogs serving as controls. The daily dosages of the various drugs required to maintain minimum blood levels of 10 to 20, 20 to 40, 40 to 60, and 60 to 80 $\mu\text{gm. \%}$ were determined

⁴ The daily diet of the dogs consisted of Purina Dog Chow Meal, and whole milk, supplemented once weekly with boiled hog liver.



FIG. 1, Plate C

FIG. 1. Plate A: Section through kidney of normal control rat 13X. Plate B: Section through cortex of same kidney. 160X. Plate C: Section through pyramid of same kidney. 160X



FIG 2 SECTION THROUGH KIDNEY OF RAT RECEIVING SULFAMERAZINE SHOWING SLIGHT DILATATION OF TUBULES IN PAPILLA, OTHERWISE NORMAL 13X

through a system of trial and error. Such dosages were then administered for periods of 30 to 38 days, one-third of the total daily dosage being administered at 8 a.m. and two-thirds at 5 p.m. The drugs, suspended in milk, were administered orally. Minimum levels of sulfonamide in the blood (i.e., those prevailing before the 8 a.m. dosage) were determined at 5-day intervals and the sulfonamide dosage adjusted where necessary to obtain the desired blood level. Blood hemoglobin and urea nitrogen determinations were made frequently.

At the end of the treatment period, the dogs were sacrificed and a complete necropsy performed. Spinal cord and ganglion, lung, heart, liver, spleen, kidney and bone marrow were studied in microscopic section.

The most important data obtained in the dog experiments are summarized in table 8. Using the change in body weight as the criterion of toxicity, sulfamethazine proved somewhat more toxic than sulfamerazine, which in turn was slightly



FIG. 3. SECTION THROUGH KIDNEY OF RAT RECEIVING SULFAMERAZINE SHOWING EXTENSIVE DILATATION OF COLLECTING TUBULES THROUGHOUT MEDULLA AND CONSIDERABLE DILATATION OF THESE TUBULES IN THE CORTX, NO GENERALIZED CELLULAR REACTION. 13X

more toxic than sulfadiazine. This is shown by the fact that Dogs 10, 18 and 25 having sulfamethazine blood levels of 40 to 45 mgm. % lost considerably more weight than Dogs 2 and 16 with similar sulfamerazine levels. Dogs 1 and 22 with corresponding sulfadiazine blood levels either showed a slight gain or no weight change.

Using renal injury as the criterion of toxicity, sulfadiazine was somewhat more toxic than sulfamerazine, which in turn was much more toxic than sulfamethazine. As was the case in the rat experiments, this latter sulfonamide appeared devoid of renal toxicity, irrespective of its concentration in the blood.

Blood level for blood level, sulfamerazine seemed to have somewhat less urinary tract toxicity in the dog than in the rat. Concretions of this drug were found in the kidney pelvis of three dogs, but in only one case (Dog 24) were they large enough or so located as to obstruct free flow of urine. In this animal, there

was slight distention of the collecting tubules in the papilla of one kidney, in other respects the kidneys were entirely normal. The low toxicity of sulfamerazine for the dog kidney was noted previously by Welch (11). Whether it bears any relation to the absence of acetylated drug in this species has not been determined.

Blood level for blood level, sulfadiazine produced distinctly more renal damage than did sulfamerazine, however, the severity of sulfadiazine lesions in these dogs was in no sense comparable to that observed in rats. All four animals with sulfadiazine blood levels of 47 mgm. % or more (Dogs 1, 15, 22 and 23) had concretions in the kidney pelvis, these deposits were much larger than those of sulfamerazine. In two of the dogs (No 1 and No 22) there was comparatively little renal pathology, the collecting tubules in the papilla being moderately distended, containing some crystalline drug and occasional polymorphous clear leucocytes. In the other animals (Dogs 15 and 23), more severe changes were noted. There was generalized dilatation of the collecting tubules throughout the kidney and more widespread cellular reaction. One of the animals (Dog 15) developed urinary retention as evidenced by a rising blood urea nitrogen content which reached 30.3 mgm. % at the time the animal was sacrificed.

Aside from the renal pathology in the dogs receiving sulfadiazine and sulfamerazine, all tissues were histologically normal, or showed only such changes as were present in the control animals as well.

3 Experiments with monkeys Twenty-seven monkeys (*Macacus rhesus*) were used in these experiments,⁴ groups of 5 received sulfadiazine, sulfamerazine or sulfamethazine, the 3 remaining animals serving as controls. Attempts were made to maintain minimum blood levels of 10, 20, 40, 60 and 80 mgm. % of the various sulfonamides. In the case of sulfamerazine and sulfamethazine this was accomplished by oral administration of a daily dosage of 0.15, 0.3, 0.6, 1.2 or 2.4 grams sulfonamide per kgm. body weight. In the case of sulfadiazine blood levels higher than 35 mgm. % could not be obtained with oral administration of a daily dose as large as 4.8 grams per kgm. body weight. In order to obtain blood levels of 40 to 60 mgm. % sulfadiazine, part of the daily dosage was administered intravenously as the sodium salt. This was done in a group of three monkeys. Irrespective of the route of administration, one third of the daily dose of sulfonamide was administered at 8 a.m., two thirds at 5 p.m. Oral administration was by stomach tube, intravenous by cephalic vein.

All monkeys receiving sulfamerazine or sulfamethazine, and 5 of those receiving sulfadiazine, were under treatment for 30 days or until death occurred. Three monkeys were treated with sulfadiazine for 50 days, receiving the drug orally during the first 30 days and both orally and intravenously during the last 20 days. Minimum levels of free and conjugated sulfonamide in blood (i.e., those prevailing before the 8 a.m. dosage) were determined at 3 to 7 day intervals. Twice during the experiment the diurnal fluctuations in blood levels were determined. Except for the monkeys receiving sulfadiazine intravenously, the blood levels of all sulfamerazine and sulfadiazine treated monkeys remained remarkably constant throughout the day; there was considerable fluctuation, however, in the blood levels of animals receiving sulfamethazine, especially in the lower dosage group (0.15, 0.3 and 0.6 grams per kgm. body weight).

Hemoglobin and urea nitrogen content of blood were determined at frequent intervals.

A complete necropsy was performed on each monkey at the end of 30 or 50 days or at death resulting from treatment. Spinal cord and ganglia, lung, heart, liver, spleen, kidney and bone marrow were studied in microscopic section.

⁴ The daily diet of the monkeys consisted of oranges, apples, carrots, potatoes, peanuts, and whole milk fortified with irradiated ergosterol.

The most pertinent data from this experiment have been summarized in table 9. As in the study with dogs, the present work suffers from use of a comparatively



FIG. 4. Plate A

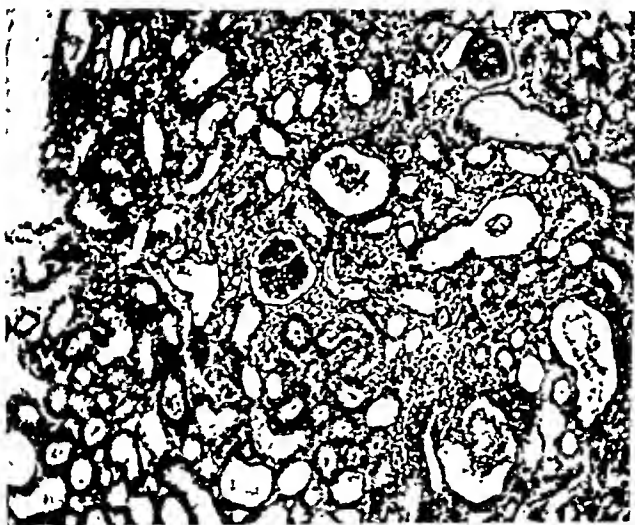


FIG. 4. Plate B

small number of animals and in addition the fact that it was impossible to maintain as high sulfadiazine levels as those of sulfamerazine and sulfamethazine.

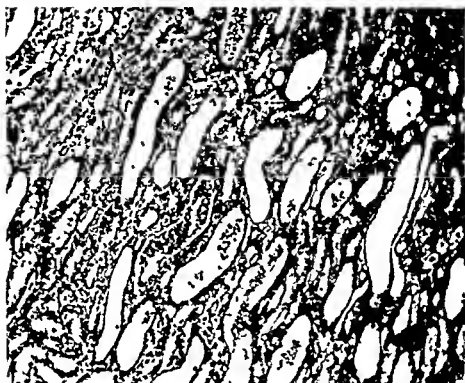


FIG 1 Plate C

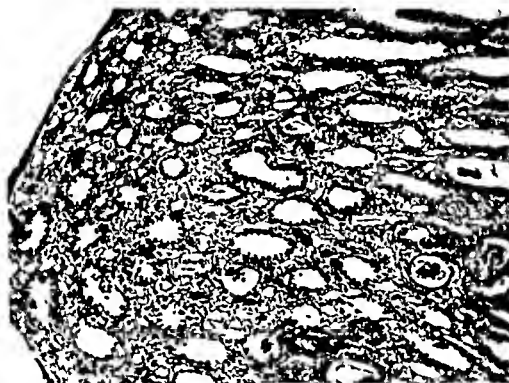


FIG 4 Plate D

FIG 4 Plate A Section through kidney of rat receiving sulfadiazine showing enor

ing tubules and in
ion through papilla
tubules leucocyte

TABLE 8

The chronic toxicity of sulfadiazine, sulfamerazine and sulfamethazine for dogs

DOG NO	SULFONAMIDE TREATMENT		DAYS OF TREATMENT	MG% PER CENT FREE SULFONAMIDE IN BLOOD*	BODY WEIGHT IN KG		REMARKS
	Grams/day/kg body weight†	Total grams during treatment			Initial	Final	
Animals receiving SD							
26	0.06-0.15	34.2	30	5.1-10.2	19.5	21.1	Sacrificed on Day 31. Urea N in blood 8.3 mgm %. Organs and tissues normal on gross and microscopic examination.
1	0.15-0.3	77.8	30	21.7-47.3	16.7	16.7	Sacrificed on Day 31. Urea N in blood 15.0 mgm %. Concretions of SD in pelvis of both kidneys, amorphous drug in both ureters and bladder, dry weight of concretions 155 mgm. Microscopically, slight injury to kidney, limited to collecting tubules of papilla, crystalline drug in tubules, few polymorphonuclear leucocytes within tubules, but no invasion of surrounding tissue. Other organs and tissues normal.
22	0.3-0.9	114.3	30	15.2-49.5	10.9	11.7	Sacrificed on Day 31. Urea N in blood 10.5 mgm %. Concretions of SD in pelvis of both kidneys, amorphous drug in left ureter and bladder, dry weight of concretions 256 mgm. Microscopically, slight injury to kidney, limited to dilatation of collecting tubules in papilla, few polymorphonuclear leucocytes within tubules, but no invasion of surrounding tissue. Other organs and tissues normal.
15	0.9	168.6	30	53.0-65.5	7.2	5.9	Sacrificed on Day 31. Urea N in blood 30.3 mgm %. Concretions in pelvis of both kidneys, ureters and in bladder, dry weight of kidney concretions 340 mgm. Microscopically, moderate injury to kidney, dilatation of collecting tubules throughout kidney, more marked in cortex than in medulla, few polymorphonuclear leucocytes within tubules but no invasion of surrounding tissue, crystalline drug deposited in calyx. Other organs and tissues normal.
23	0.3-1.2	308.9	38	49.2-100	14.9	12.7	Sacrificed on Day 39. Urea N in blood 16.5 mgm %. Concretions in pelvis of both kidneys, dry weight of concretions 503 mgm. Microscopically, moderate injury to kidney, dilatation of collecting tubules throughout kidney. A few dilated tubules contained many polymorphonuclear leucocytes with invasion of intertubular tissue in some cases. Other organs and tissues normal.
Animals receiving SMD							
20	0.03-0.12	95.4	30	1.3-16.2	17.2	18.1	Sacrificed on Day 31. Urea N in blood 9.0 mgm %. Organs and tissues normal on gross and microscopic examination.
16	0.09-0.24	132.1	30	21.7-50	12.7	11.3	Sacrificed on Day 31. Urea N in blood 10.6 mgm %. Organs and tissues normal on gross and microscopic examination.
2	0.09-0.21	156.6	30	23.6-37.4	13.6	13.6	Sacrificed on Day 31. Urea N in blood 7.5 mgm %. Small concretions of SMD in pelvis of right kidney, dry weight of concretions 19 mgm. Organs and tissues normal on gross and microscopic examination.

* Blood levels shown are the lowest and highest concentrations determined 15 hours after the p.m. dose, presumably these are the minimum blood levels.

† First figure indicates starting dose, second shows dose required to maintain highest blood level.

TABLE 8—Continued

DOG NO	SULFONAMIDE TREATMENT		DAYS OF TREATMENT	MG% FREE SULFONAMIDE IN BLOOD ^a	BODY WEIGHT IN KG		REMARKS
	Grams/day/kg body weight	Total grams during treatment			Initial	Final	
Animal receiving SMD—Continued							
24	0.9-1.2	240.8	38	42.3-61.4	9.9	5.9	Sacrificed on Day 39. Urea N in blood 14.1 mgm %. Small concretions of SMD in pelvis of right kidney, dry weight of concretions 10 mgm. Slight renal injury characterized by deposits of drug in calyx with resulting distention of collecting tubules of papilla. Other organs and tissues normal on gross and microscopic examination.
5	0.9-1.2	293.2	38	49.1-64.4	11.7	7.7	Sacrificed on Day 39. Urea N in blood 8.3 mgm %. Small concretion of SMD in pelvis of right kidney, dry weight of concretion 9 mgm. Organs and tissues normal on gross and microscopic examination.
Animals receiving SMD							
7	0.15-0.27	134.0	38	10.5-23.3	18.3	19.9	Sacrificed on Day 39. Urea N in blood 8.8 mgm %. Organs and tissues normal on gross and microscopic examination.
10	0.3-0.6	192.0	38	15.1-44.0	15.8	13.1	Sacrificed on Day 39. Urea N in blood 8.4 mgm %. Organs and tissues normal on gross and microscopic examination.
18	0.3-0.9	278.9	38	25.4-40.4	11.7	8.6	Sacrificed on Day 39. Urea N in blood 12.2 mgm %. Organs and tissues normal on gross and microscopic examination.
25	0.7-0.9	291.8	38	23.2-44.4	11.3	7.7	Sacrificed on Day 39. Urea N in blood 12.6 mgm %. Organs and tissues normal on gross and microscopic examination.
4	0.7-1.5	373.9	38	33.5-74.6	10.9	7.2	Sacrificed on Day 39. Urea N in blood 14.3 mgm %. Organs and tissues normal on gross and microscopic examination.
Controls							
6			34		9.9	10.0	Sacrificed on Day 34. Urea N in blood 11 mgm %. Organs and tissues normal on gross and microscopic examination.
27			35		11.7	12.2	Sacrificed on Day 35. Urea N in blood 14 mgm %. Organs and tissues normal on gross and microscopic examination.
17			37		9.5	9.5	Sacrificed on Day 37. Urea N in blood 9.0 mgm %. Organs and tissues normal on gross and microscopic examination.

Nevertheless, the data do permit one to conclude: first, that blood level for blood level there is no significant difference in the overall toxicity of the three drugs as indicated by weight changes, secondly, that the only specific pathological lesions attributable to any of the compounds are those found in the kidney, thirdly, that sulfamethazine appears to be devoid of renal toxicity for the monkey just as for the rat and dog, and lastly, that sulfamerazine is capable of producing a severe renal lesion in the monkey, although relatively enormous blood levels must be maintained to accomplish this.

TABLE 9

The chronic toxicity of sulfadiazine, sulfamerazine and sulfamethazine for monkeys

MONKEY NO.	SULFONAMIDE TREATMENT		DAYS OF TREATMENT	MG. PER CENT SULFONAMIDE IN BLOOD*		BODY WEIGHT IN KG.		REMARKS
	Grams/day/kg. body weight†	Total grams during treatment		Free	Conjugated	Initial	Final	
Animals receiving SD								
934	0.3-0.6	66.4	30	7.3-19.5	0	4.04	3.93	Sacrificed on Day 31. Urea N in blood 11 mgm. %. Organs and tissues normal on gross and microscopic examination.
14	0.3-0.6	30.7	30	6.6-14.0	0	1.75	2.03	Sacrificed on Day 31. Urea N in blood 13.5 mgm. %. Organs and tissues normal on gross and microscopic examination.
13	0.6-1.2	94.0	30	23.7-40.0	0-8.3	3.20	3.25	Sacrificed on Day 31. Urea N in blood 8 mgm. %. Organs and tissues normal on gross and microscopic examination.
6	1.2-2.4	154.0	30	14.9-32.8	0-3.5	2.50	2.86	Sacrificed on Day 31. Urea N in blood 7.5 mgm. %. Organs and tissues normal on gross and microscopic examination.
12	2.4-4.8	341.8	30	26.4-35.4	0-2.6	2.80	2.76	Sacrificed on Day 31. Urea N in blood 10.5 mgm. %. Slight renal pathology consisting of precipitation of drug in collecting tubules of isolated areas of cortex and medulla with resulting dilatation; little cellular reaction. Other organs normal.
30	0.6-5.0	316.8	50	3.6-60.0	0-3.0	2.44	2.6	Sacrificed on Day 51. Urea N in blood 9.3 mgm. %. Moderate renal pathology consisting of precipitation of drug in collecting tubules in many areas of cortex and medulla; dilatation of tubules marked; moderate infiltration of polymorphonuclear leucocytes. Other organs normal.
942	1.2-5.0	527.5	50	21.5-55.5	0-6.0	3.18	3.25	Sacrificed on Day 51. Urea N in blood 18.5 mgm. %. Kidneys grossly enlarged. Moderate renal pathology, somewhat more extensive than in M 30; precipitated drug in collecting tubules in cortex and medulla resulting in generalized dilatation; infiltration of tubules and surrounding connective tissue with polymorphonuclear leucocytes. Other organs normal.
25	2.4-5.0	554.0	50	17.8-42.2	0-4.1	2.79	3.05	Sacrificed on Day 51. Urea N in blood 10.3 mgm. %. Organs and tissues normal on gross and microscopic examination.
Animals receiving SMD								
29	0.15-0.3	15.5	30	8.9-11.9	0	1.97	2.26	Sacrificed on Day 31. Urea N in blood 6.5 mgm. %. Organs and tissues normal on gross and microscopic examination.
947	0.15-0.3	30.4	30	8.4-14.6	0	3.88	4.07	Sacrificed on Day 31. Urea N in blood 10.5 mgm. %. Organs and tissues normal on gross and microscopic examination.
11	0.3-0.6	38.0	30	18.9-28.8	0	2.36	2.51	Sacrificed on Day 31. Urea N in blood 16.5 mgm. %. Organs and tissues normal on gross and microscopic examination.

* Blood levels shown are the lowest and highest concentrations determined 15 hours after the p.m. dose; presumably these are the minimum blood levels.

† First figure indicates starting dose; second shows dose required to maintain highest blood level.

TABLE 9—Continued

MONKEY NO	SULFONAMIDE TREATMENT		DAYS OF TREATMENT	MG% PER CENT SULFONAMIDE IN BLOOD*		BODY WEIGHT IN KG		REMARKS
	Grams/day/kg body weight	Total grams during treatment		Free	Conjugated	Initial	Final	
Animals receiving SMD—Continued								
941	0.3-0.6	54.5	30	23.6-37.0	0	3.29	3.55	Sacrificed on Day 31. Urea N in blood 8.5 mgm %. Organs and tissues normal on gross and microscopic examination.
21	0.6-1.2	81.0	30	24.2-36.1	0	2.55	3.02	Sacrificed on Day 31. Urea N in blood 12.5 mgm %. Organs and tissues normal on gross and microscopic examination.
15	0.6-1.2	91.1	30	43.5-55.6	27.4-45.4	3.18	2.43	Sacrificed on Day 31. Urea N in blood 97.5 mgm %. Kidneys grossly enlarged. Severe and extensive renal pathology, most marked in collecting tubules of medulla and cortex, but involving convoluted tubules as well although to a lesser degree. Collecting tubules greatly dilated, many filled with polymorphonuclear leucocytes and residue of drug. Tubular epithelium degenerated in some areas in others stretched thin. Polymorphonuclear leucocytes invading surrounding tissue to point where kidney structure no longer clear. Thrombi forming in interlobular veins. Congestion evident in spleen. Fatty degeneration in liver. Other tissues and organs normal on microscopic examination.
0	1.2-2.4	70.0	13	63.5-65.3	50.5-112.4	2.72	2.65	Died on Day 13. Rupture left ureter. Free fluid in body cavity. Concretions of drug in both ureters also large amount of amorphous drug. Kidneys grossly enlarged. Severe renal pathology, most marked in collecting tubules of medulla but also extending into cortex. Marked dilatation of collecting tubules with extensive infiltration with polymorphonuclear leucocytes.
20	1.2-2.4	101.3	17	49.5-75.5	45.5-77.2	2.95	2.25	Died on Day 17. Kidneys grossly enlarged. Ureters filled with drug preventing flow of urine. Severe and extensive renal pathology involving collecting tubules in both cortex and medulla. Marked dilatation of collecting tubules polymorphonuclear leucocytes infiltrating tubules and surrounding connective tissue.
Animals receiving SMMD								
23	0.15-0.3	18.5	30	1.0-6.3		2.25	2.31	Sacrificed on Day 30. Urea N in blood 11 mgm %. Organs and tissues normal on gross and microscopic examination.
26	0.15-0.3	32.6	30	6.1-10.7		3.9	3.7	Sacrificed on Day 30. Urea N in blood 11 mgm %. Organs and tissues normal on gross and microscopic examination.

TABLE 9—Continued

MONKEY NO.	SULFONAMIDE TREATMENT		DAYS OF TREATMENT	MG. PER CENT SULFONAMIDE IN BLOOD*		BODY WEIGHT IN KG.		REMARKS
	Grams/day/kg. body weight	Total grams during treatment		Free	Conjugated	Initial	Final	
Animals receiving SMMD—Continued								
5	0.3-0.6	35.2	30	6.3-22.4		2.36	2.23	Sacrificed on Day 30. Urea N in blood 11 mgm. %. Organs and tissues normal on gross and microscopic examination.
945	0.3-0.6	55.6	30	6.9-16.6		3.64	3.50	Sacrificed on Day 30. Urea N in blood 15 mgm. %. Organs and tissues normal on gross and microscopic examination.
19	0.6-1.2	79.0	30	15.8-32.5	8.4- 23.2	2.57	2.40	Sacrificed on Day 30. Urea N in blood 12 mgm. %. Organs and tissues normal on gross and microscopic examination.
8	0.6-1.2	101.5	30	22.8-55.8	7.3- 0.4	3.14	2.96	Sacrificed on Day 30. Urea N in blood 8 mgm. %. Organs and tissues normal on gross and microscopic examination.
16	1.2-2.4	172.7	30	41.6-85.4	15.0- 16.9	3.0	2.58	Sacrificed on Day 30. Urea N in blood 9.5 mgm. %. Organs and tissues normal on gross and microscopic examination.
2	1.2-2.4	71.2	14	86.5-93.7	13.5- 34.0	2.63	2.29	Died on Day 14. Lower segments of small intestine hemorrhagic; microscopic involvement of both mucosa and submucosa. Other organs and tissues normal on gross and microscopic examination.
Controls								
17			26			1.82	2.54	Sacrificed on Day 26. Urea N in blood 0.5 mgm. %. Organs and tissues normal on gross and microscopic examination.
4			27			1.62	1.52	Sacrificed on Day 27. Urea N in blood 13.5 mgm. %. Organs and tissues normal on gross and microscopic examination.
3			49			4.15	4.54	Sacrificed on Day 49. Urea N in blood 10.8 mgm. %. Organs and tissues normal on gross and microscopic examination.

All of the monkeys receiving sulfadiazine survived the treatment periods, maintained their weight, and were in excellent condition when sacrificed. Six of the eight animals had blood levels of 14 to 42 mgm. % free sulfadiazine and 0 to 8.3 mgm. % conjugated drug. One of these six monkeys (No. 12, free sulfadiazine in blood 26 to 35 mgm. %) had slight renal lesions, as described in the table. The other five monkeys were entirely normal. Two other animals (No. 30 and No. 942) had peak blood levels of free sulfadiazine of 60 and 56 mgm. % respectively, with maximum conjugated drug levels of 3 and 6 mgm. %. These animals had more severe renal lesions than those in Monkey 12 or in the dogs receiving sulfadiazine, but less severe than in several of the monkeys receiving sulfamerazine. The blood urea nitrogen content of Monkey 30 increased during the terminal phases of the experiments, but only slightly exceeded the normal levels for this species.

Of the eight monkeys receiving sulfamerazine, two died during treatment, No.

0 on the 13th day and No. 20 on the 17th. The free sulfonamide levels in the blood of these animals ranged from 63.5 to 65.3 and from 19.5 to 75.8 mgm % respectively, the conjugated drug levels from 59.5 to 112.4 and from 46.5 to 77.2 mgm %. Monkey 0 died comparatively suddenly, necropsy showed a ruptured left ureter apparently resulting from occlusion of the ureter with sulfamerazine concretions. (The concretions consisted of both free and conjugated drug.) Monkey 20 showed a more gradual downhill course. Both monkeys had severe renal lesions, comparable to those seen in the rats receiving sulfadiazine and much more severe than those in the monkeys receiving this latter sulfonamide. All other organs and tissues were normal on both gross and microscopic examination.

Six monkeys survived 30 days treatment with sulfamerazine. Five of these animals had blood levels of free sulfamerazine of 12 to 37 mgm %. All of these monkeys were in as good condition as were those with similar blood levels of sulfadiazine. None had lesions in the kidney or other organs. The sixth monkey (No. 15) was in a debilitated condition at the end of the experiment. During the course of treatment the blood level of free sulfamerazine ranged from 13.8 to 58.6 mgm % whereas that of conjugated drug varied from 27.4 to 48.4 mgm %. The blood urea nitrogen content rose progressively and was 97.5 mgm % when the animal was sacrificed. The renal lesions in this monkey were more severe than any others encountered in the entire study (figure 5). Both kidneys were grossly enlarged weighing 28 and 29.5 grams respectively as contrasted with weights of 8.0 and 9.0 grams for the kidneys of control Monkey 17 and weights of 10 and 11.5 grams for control Monkey 3. The ureters were not obstructed and the kidney pelvis were free of drug. The collecting tubules were enormously dilated, the distention being greatest in the pyramid but severe in cortex and papilla as well. Many of the collecting tubules were filled with crystalline sulfonamide, others contained polymorphonuclear leucocytes and cellular debris. In some places the tubular epithelium was intact but in many places it had degenerated. In some areas tubules had been replaced with fibrous tissue. In some areas of the cortex the convoluted tubules were markedly distended, where this occurred the renal corpuscles appeared compressed and avascular, but otherwise normal. Of the other organs only the spleen and liver were abnormal. The conditions in liver and spleen reflected the profound toxemia that existed during the last 7 days of the treatment period.

Of the eight monkeys receiving sulfamethazine, one (No. 2) died on the 14th day, the remaining seven survived the experimental period. The blood levels in Monkey 2 ranged from 87 to 94 mgm % free drug and from 13.5 to 34 mgm % conjugated drug. This animal had a steady downhill course from the 9th to the 14th day, being in coma during the last 36 hours. The necropsy and microscopic examination yielded entirely negative findings except in the small intestine where many punctate hemorrhages were found.

Of the seven monkeys that survived 30 days treatment with sulfamethazine, six were in excellent condition at the end of the experiment. In these six animals the blood levels of free sulfamethazine ranged between 6 and 56 mgm %, the levels of conjugated drug ranged between 8 and 23 mgm %. The seventh

monkey lost a significant amount of weight during the experiment and became increasingly inactive; in this animal the level of free sulfamethazine reached 85

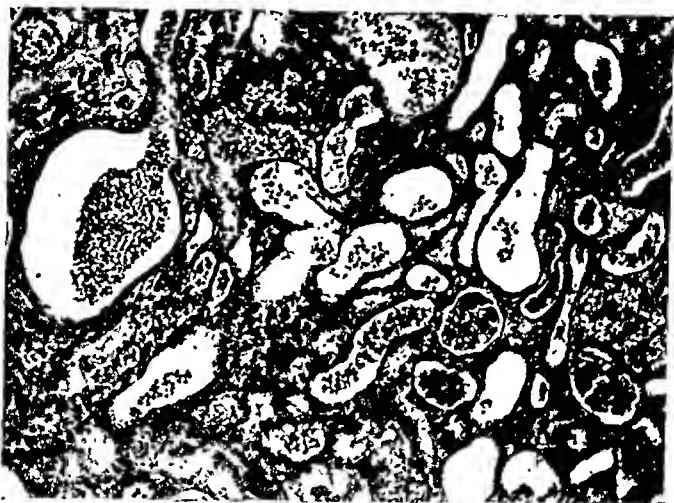


FIG. 5. Plate A



FIG. 5. Plate B

FIG. 5. Plate A: Section through cortex of kidney of Monkey 15, receiving sulfamerazine. Note enormous dilatation of collecting tubules, accumulation of polymorphonuclear leucocytes and normal glomeruli. 160X. Plate B: Section through pyramid of same kidney, showing dilatation of collecting tubules and cellular reaction. 160X.

mgm. %. The necropsy on this animal was negative. Renal lesions were absent in all monkeys receiving sulfamethazine.

Finally, attention should be called to the absence of neuropathology in all the chronic toxicity studies. Welch (11) has pointed out that the nerve damaging potentialities of sulfamerazine and sulfamethazine for the chick are no greater than those of sulfadiazine. This observation is supported by the results of the present study and needs special mention in view of the tendency to regard all methyl sulfonamides as potential producers of nerve injury.

COMMENTS AND SUMMARY

The data in the present study give a fairly clear picture of the relative toxicities of sulfamerazine, sulfamethazine and sulfadiazine. With respect to acute toxicity, there is little to choose between the drugs on the basis of lethal doses and associated blood levels. When administered parenterally (but not orally) sulfamethazine was somewhat more toxic than the other drugs. Acetylsulfamethazine was slightly less toxic, however. In the acute toxicity tests, sulfamerazine and sulfamethazine killed mice more rapidly than sulfadiazine; in this respect, the former drugs resembled sulfathiazole and sulfapyridine. Sulfadiazine seems to be unique among the sulfonamides in producing delayed deaths.

As judged by effects on the weight of growing and mature animals there is no striking difference in the "over-all" chronic toxicity of sulfamerazine, sulfamethazine and sulfadiazine. These drugs had essentially the same adverse effects on the growth of immature rats, when comparable blood levels were maintained. Likewise they had nearly identical effects on the body weight of mature dogs and monkeys. Blood level for blood level, sulfamethazine appeared to have a slightly greater effect on the weight of the dog than sulfamerazine and sulfadiazine. In view of the small number of observations, too much importance should not be attached to this difference.

There are striking differences, however, in the renal toxicity of these sulfonamides. Sulfamethazine seems to be devoid of renal toxicity, irrespective of the concentration of this drug in the blood. Sulfamerazine has decidedly less renal toxicity than sulfadiazine. Differences in tubular reabsorption and toxicity of the drugs for the tubular epithelium may be partially responsible for the observed differences in renal toxicity. The most important factor, however, is probably the solubility of the drugs in acid urine, sulfamethazine, the most soluble drug, having the least toxicity and sulfadiazine, the least soluble drug, having the most toxicity. This explanation is particularly favored by experiments (17) which show that when the solubility differences are eliminated by alkalization of the urine, sulfadiazine has almost as low renal toxicity as sulfamethazine.

In view of their low renal toxicity, both sulfamerazine and sulfamethazine would appear to be useful additions to the list of sulfonamide drugs. This conclusion assumes of course that the results of clinical toxicity studies corroborate the above experimental findings and that the antibacterial activities of these new sulfonamides compare favorably with those of the now widely used drugs.

CONCLUSIONS

In so far as acute and "over-all" chronic toxicity are concerned there seems little to choose between sulfamerazine, sulfamethazine and sulfadiazine. How-

ever, in so far as solubility and renal toxicity are concerned (and the latter may be largely a function of the former) both sulfamerazine and sulfamethazine seem to be more desirable drugs than sulfadiazine. Admittedly other factors, such as antibacterial activity and ease of maintaining effective blood levels in man, must be considered before the place of sulfamerazine and sulfamethazine in sulfonamide therapy can be determined.

We are indebted to Elizabeth Cooper, Suzanne Weisz and Kathryn Weichert for technical assistance, to Mr. Joseph B. Homan, Professor of Medical Art, College of Medicine, University of Cincinnati, for preparation of the photomicrographs, and to Katherine Bachman for preparation of this manuscript.

REFERENCES

- (1) ROBLIN, R. O., JR., J. H. WILLIAMS, P. S. WINNEK AND J. P. ENGLISH, *J. Amer. Chem. Soc.*, **62**: 2002, 1940.
- (2) CALDWELL, W. T., E. C. KORNFELD AND C. K. DONNELL, *J. Amer. Chem. Soc.*, **63**: 2188, 1941.
- (3) SPRAGUE, J. M., L. W. KISSINGER AND R. M. LINCOLN, *J. Amer. Chem. Soc.*, **63**: 3028, 1941.
- (4) ROBLIN, R. O., JR., P. S. WINNEK AND J. P. ENGLISH, *J. Amer. Chem. Soc.*, **64**: 567, 1942.
- (5) FELLOWS, E. J., *Proc. Soc. Exper. Biol. and Med.*, **48**: 680, 1941.
- (6) ROSE, F. L., A. R. MARTIN AND H. G. L. BEVAN, *This Journal*, **77**: 127, 1943.
- (7) FEINSTONE, W. H., R. H. FOLLIS AND R. D. WILLIAMS, Unpublished observations.
- (8) BRATTON, A. C., AND E. K. MARSHALL, JR., *J. Biol. Chem.* **128**: 537, 1939.
- (9) GILLIGAN, D. R., AND N. PLUMMER, *Proc. Soc. Exper. Biol. and Med.*, **53**: 142, 1943.
- (10) JENSEN, O. J., JR., AND C. L. FOX, JR., *J. Urol.*, **49**: 334, 1943.
- (11) WELCH, A. D., P. A. MATTIS, A. R. LATVEN, W. M. BENSON AND E. H. SHIELS, *This Journal*, **77**: 357, 1943.
- (12) MACARTNEY, D. W., G. S. SMITH, R. W. LUXTON, W. A. RAMSAY AND J. GOLDMAN, *Lancet*, **1**: 639, 1942.
- (13) Personal communication to Welch et al. (11).
- (14) GOODWIN, R. A., JR., O. L. PETERSON AND M. FINLAND, *Proc. Soc. Exper. Biol. and Med.*, **51**: 262, 1942.
- (15) FEINSTONE, W. H., R. D. WILLIAMS, R. T. WOLFF, E. HUNTINGTON AND M. L. CROSSLLEY, *Bull. Johns Hopkins Hosp.*, **67**: 427, 1940.
- (16) POWELL, H. M., AND K. K. CHEN, *J. Indiana. M. A.*, **34**: 602, 1941.
- (17) HUGHES, H. B., AND L. H. SCHMIDT, Observations to be published.

THE CHEMOTHERAPEUTIC ACTIVITIES OF SULFAMERAZINE AND SULFAMETHAZINE*

L H SCHMIDT, CLARA L SESLER AND HETTIE B HUGHES

From the Institute for Medical Research, Christ Hospital, Cincinnati, Ohio

Received for publication December 13, 1943

Pharmacological studies (1) have shown that in certain respects sulfamerazine and sulfamethazine have less toxicity than sulfadiazine and have suggested that from this standpoint at least the former compounds might be more desirable therapeutic agents. Preliminary clinical and experimental observations (2-15) have indicated that sulfamerazine and sulfamethazine possess appreciable antibacterial activity. Before the place of these compounds in sulfonamide therapy can be determined, however, there must be a comprehensive evaluation of their activities against different bacterial species and a critical comparison of their effectiveness with that of the better established drugs. To this end, a study was made of the activities of sulfamerazine and sulfamethazine against pneumococci, β hemolytic streptococci, staphylococci, Friedlander's bacilli, dysentery bacilli and *Escherichia coli*. In most instances *in vivo* activities were compared with those of sulfadiazine, *in vitro* activities were compared with those of sulfadiazine and sulfathiazole.

EXPERIMENTAL

In vivo activity

METHODS

Experiments in mice The activities of sulfamerazine, sulfamethazine and sulfadiazine¹ were compared against infections with two different strains each of pneumococci, β hemolytic streptococci, staphylococci, Friedlander's bacilli and dysentery bacilli.² The strains selected were of different sulfonamide sensitivity and were chosen so that the activities of the compounds could be observed against both a comparatively sensitive and a comparatively resistant strain. The pneumococci, β hemolytic streptococci, Friedlander's bacilli and staphylococci had undergone repeated mouse passages. Their virulence was constant and their response to the sulfonamides had been well established in previous experiments. The dysentery bacilli were cultured in semi solid agar medium according to the techniques of Cooper and Keller (16), the stock cultures were stored in the refrigerator.

White mice, weighing 13 to 16 grams, were used throughout the experiments. They were infected intraperitoneally, groups of 30 being treated with the various drugs and 20 serving

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Institute for Medical Research, Christ Hospital, Cincinnati, Ohio.

¹ The sulfadiazine, sulfamerazine and sulfamethazine used in this study were supplied through the generosity of Dr. W. A. Feiler, Sharp and Dohme, Inc., Philadelphia, Pa., and Dr. R. O. Roblin, Jr., American Cyanamid Company, Stamford, Conn.

² We are indebted to Dr. H. M. Powell, Eli Lilly and Co., Indianapolis, Ind., for the 679 strain and to Dr. Wm. Vervey, Sharp and Dohme, Inc., for the Smith strain of *Staphylococcus aureus*. Dr. Merlin Cooper, Children's Hospital Research Foundation, Cincinnati, Ohio, kindly provided the Bennett and Chertam strains of dysentery bacilli.

as untreated controls. The sulfonamides, suspended in 10% aecia, were administered by stomach tube in the doses and at the intervals indicated below. All mice not succumbing to the infection were observed for 30 days.

Preliminary experiments were carried out with each organism to determine the dose of sulfadiazine and the duration of treatment required to protect 50 ± 20 per cent of the mice. In one series of experiments, all three drugs were administered in this same dosage. In a second series of experiments, the doses of sulfamerazine and sulfamethazine were adjusted

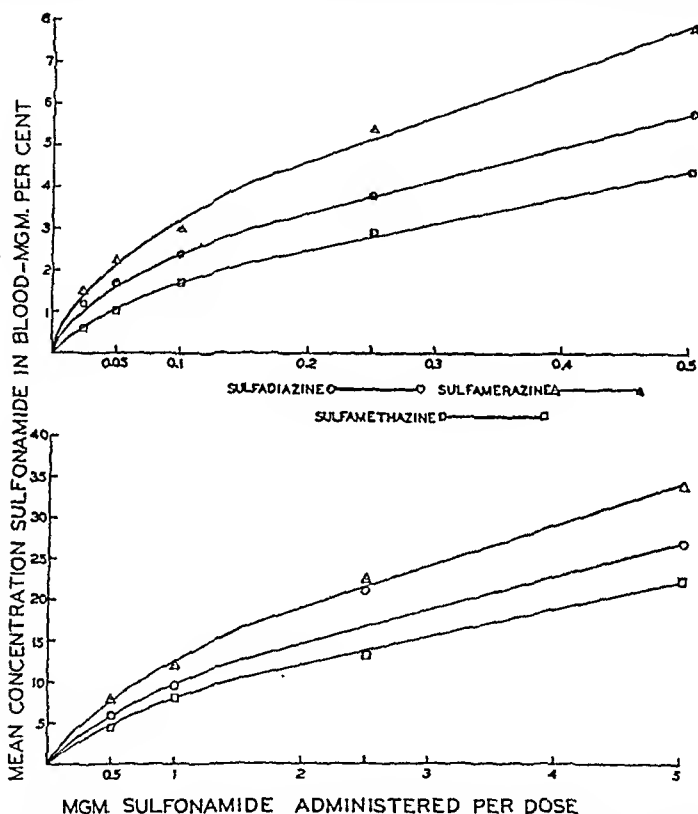


FIG. 1. CONCENTRATIONS OF SULFAMERAZINE, SULFAMETHAZINE AND SULFADIAZINE IN THE BLOOD OF MICE RECEIVING VARIOUS DOSES OF THESE DRUGS, ADMINISTERED AS IN THE THERAPEUTIC TESTS

so that the levels of these drugs in the blood were approximately equal to those of sulfadiazine. These latter doses were determined from the data presented in figure 1.³

³ Figure 1 was prepared from data obtained as follows. Groups of 6 white mice (13 to 16 grams) were treated with the indicated doses of sulfonamides according to the schedule used in treatment. Four hours after receiving the seventh dose, the mice were sacrificed. The free sulfonamide content of the blood of each animal was determined, and the results averaged for each dosage group. The resulting data have been termed the "mean" sulfonamide content of the blood; perhaps a more correct designation would be the average concentration prevailing midway in the treatment period.

Pneumococcal infections The pneumococci used for infecting the mice were derived from a 12 to 14 hour blood broth culture prepared from the heart blood of a passage mouse. This culture was diluted serially in beef infusion broth, one cc of a 10^{-4} dilution was used as the infecting dose. The mice were treated 2, 8, and 14 hours after infection and every 8 hours thereafter for five additional days.

β hemolytic streptococcal infections The organisms used for infecting the mice were prepared in the same manner as the pneumococci. The mice were treated 2, 8, and 14 hours after infection and every 8 hours thereafter for five additional doses.

Friedlander's bacillus infections The Friedlander's bacilli used for infecting the mice were derived from a 12 to 14 hour blood broth culture prepared from the heart blood of a passage mouse. This culture was diluted serially in beef infusion broth, one cc of $1/50,000$ dilution was used as the infecting dose. The mice were treated at the same intervals and for the same period as those infected with pneumococci.

Staphylococcus aureus infections The infecting dose of staphylococci was prepared from a 12 to 14 hour agar slant culture which had been inoculated from a mouse passage culture. The organisms from the agar slant were washed off with distilled water, and diluted to a turbidity equivalent to 500,000,000 organisms per cc. This suspension was diluted serially to 10^{-4} , the final dilution being made in freshly prepared Armour's mucin (3% concentration, pH 7.4). The infecting dose was 0.5 cc of this 10^{-4} dilution. The mice were treated at the same intervals and for the same period as those infected with β hemolytic streptococci. Mice surviving 30 days were sacrificed and the presence of abscesses in the kidneys and visceral organs was noted.

Dysentery bacillus infections The infecting dose of dysentery bacilli was prepared in the following manner. One cc of stock culture was transferred to beef infusion broth and incubated for 6 hours, one cc of the resulting culture was subcultured in broth and incubated for 20 hours. This culture was diluted to 10^{-4} in beef infusion broth, and from there to 10^{-4} in a 3% solution of Wilson's Bacteriological Mucin⁴ (freshly prepared solution, pH 7.4). 0.5 cc of this dilution served as the infecting dose. The mice were treated with sulfonamide at the same intervals and for the same period as those infected with β hemolytic streptococci.

Experiments in rats The relative effectiveness of sulfamerazine, sulfamethazine, sulfadiazine, and sulfathiazole against pneumococcal meningitis in the rat was also studied. Only the McGovern strain of pneumococcus was used in these experiments. White rats, Sprague-Dawley strain, weighing 70 to 110 grams were infected intracranially with 0.1 cc of a 10^{-4} dilution of a 12 to 14 hour blood broth culture prepared from the heart blood of a passage mouse. The rats were anaesthetized lightly with ether. The heads of the rats were swabbed with tincture of mercuric iodine and the diluted culture was injected through the foramen magnum occipitale. 20 or 40 mgm doses of the various sulfonamides, suspended in water, were administered by stomach tube 2 and 8 hours after infection and at 8 A.M. and 4 P.M. thereafter for four additional days. Necropsies were performed on representative animals that died during the course of the experiment. Spinal fluid smears and blood cultures being prepared and examined for pneumococci. At the end of 30 days the surviving animals were sacrificed, cerebrospinal fluid was cultured and the brain was removed, fixed in formalin and sectioned for microscopic study.

RESULTS *Experiments in mice* Throughout the experiments with mice, both curative and life prolonging activities have been considered in evaluating the efficacy of the 3 drugs.

Pneumococcal infections The results of the experiments summarized in table 1, indicate that sulfamerazine and sulfamethazine have slightly greater activity than sulfadiazine against pneumococcal infections in mice. This was shown in the experiments in which equal doses of the drugs were administered (cf. 2.5 mgm

⁴ Wilson's Bacteriological Mucin was used after it was found that Armour's Concentrated Mucin would not enhance the infectivity of our strains of dysentery bacilli.

dose experiments) as well as those in which equal concentrations of the drugs were maintained in the blood. It should be noted, however, that the differences in activity were comparatively small and scarcely beyond the experimental error of the test. Nevertheless, the fact that sulfadiazine was consistently less effective

TABLE 1

Comparative effects of sulfadiazine, sulfamerazine and sulfamethazine on pneumococcal infections in mice

STRAIN	NO. OF ORGANISMS IN INFECTING DOSE	DRUG*	DOSE† OF DRUG	AVER. CONC. OF DRUG IN BLOOD‡	NO. OF MICE INFECTED	DEATHS ON DAY										AVER. HOURS SURVIVAL OF MICE THAT DIED	SURVIVORS	
						1	2	3	4	5	6	7-10	11-20	21-30	No.		%	
Equal doses of drug																		
McGovern (type I)	540	None	mgm.	mgm. %	20	8	12	0	0	0	0	0	0	0	26	0	0	
		SD	0.5	5.8	30	0	2	10	4	6	3	4	1	0	100	0	0	
		SMD	0.5	7.8	29	0	8	5	3	7	0	6	0	0	93	0	0	
		SMMD	0.5	4.4	30	0	8	18	1	2	1	0	0	0	60	0	0	
		SD	2.5	19.0	30	0	1	4	2	4	2	2	0	0	103	15	50	
		SMD	2.5	22.0	29	0	0	0	3	0	0	3	3	0	202	20	69	
		SMMD	2.5	13.5	30	0	1	0	0	2	2	8	0	0	148	17	57	
SV-1 (type I)	270	None			19	0	19	0	0	0	0	0	0	0	37	0	0	
		SD	0.5	5.8	29	0	1	10	3	3	4	7	0	0	107	1	3	
		SMD	0.5	7.8	30	0	0	7	8	7	4	3	0	0	99	1	3	
		SMMD	0.5	4.4	30	0	4	6	5	6	3	5	1	0	107	0	0	
		SD	2.5	19.0	30	0	0	1	1	1	0	7	1	0	174	19	63	
		SMD	2.5	22.0	30	0	0	1	0	0	0	2	2	0	209	25	83	
		SMMD	2.5	13.5	30	0	0	0	0	1	1	3	0	0	155	25	83	
Equal concentrations of drug in blood																		
McGovern (type I)	850	None			20	20	0	0	0	0	0	0	0	0	22	0	0	
		SD	2.5	19.0	30	0	0	0	0	0	0	10	2	0	203	18	60	
		SMD	2.5	22.0	30	0	0	0	0	0	0	0	5	0	321	25	83	
		SMMD	5.0	22.5	30	0	0	1	0	0	0	0	1	1	351	27	90	

* SD = sulfadiazine; SMD = sulfamerazine; SMMD = sulfamethazine.

† These doses were administered 2, 8, 14 and 22 hours after infection and every 8 hours thereafter for 5 additional days.

‡ These values are the concentrations of drug occurring in the blood at the time midway between treatments, i.e. 4 hours after dosage.

tive than sulfamerazine and sulfamethazine does suggest that the latter are slightly more effective drugs against pneumococcal infections.

β hemolytic streptococcal infections: The results of the experiments with *β* hemolytic streptococci have been summarized in table 2. The data show that there was no significant difference in the activities of the drugs against infections

with strain C203, however, against strain CF1, sulfadiazine was slightly but consistently more active than sulfamethazine, which in turn was somewhat more active than sulfamerazine. More of the mice treated with sulfadiazine recovered than of those treated with sulfamethazine or sulfamerazine. Of the mice that did not recover, those receiving sulfamerazine died much sooner than those

TABLE 2

Comparative effects of sulfadiazine, sulfamerazine and sulfamethazine on beta hemolytic streptococcal infections in mice

STRAIN	NO OF ORGANISMS IN INFECTING DOSE	DILG*	DOSE OF DRUG†	AVER CONC OF DRUG IN BLOOD‡	NO OF MICE INFECTED	DEATHS ON DAY												AVER HOURS SURVIVAL OF MICE THAT DIED	SURVIVORS	
						1	2	3	4	5	6	7	10	11	20	21	30		No	%
Equal doses of drug																				
C203	225	None	mgm	mgm %	20	16	4	0	0	0	0	0	0	0	0	0	24	0	0	
		SD	0.2	3.6	30	1	0	0	1	2	7	3	3	0	0	0	170	13	43	
		SMD	0.2	5.0	30	0	0	0	0	0	2	11	0	0	0	0	179	17	57	
		SMMD	0.2	2.6	30	0	0	0	0	5	6	2	0	1	1	3	137	16	53	
CF #1	106	None			20	15	4	0	0	0	0	0	1	0	0	39	0	0		
		SD	0.05	1.6	29	0	0	0	0	0	0	1	1	0	0	0	210	27	93	
		SMD	0.05	2.2	29	0	0	0	1	0	2	7	0	0	0	0	165	10	65	
		SMMD	0.05	1.0	30	0	0	0	1	1	1	1	3	2	0	0	287	21	70	
Equal concentrations of drug in blood																				
C203	193	None			20	20	0	0	0	0	0	0	0	0	0	0	20	0	0	
		SD	0.1	2.4	30	0	0	0	1	3	7	4	0	0	0	0	135	15	50	
		SMD	0.05	2.2	30	0	3	0	0	4	6	5	1	0	0	0	132	11	37	
		SMMD	0.2	2.0	30	0	0	0	1	1	2	3	0	0	0	0	126	13	43	
CF #1	89	None			20	15	2	0	0	0	0	0	0	0	0	24	3	15		
		SD	0.05	1.6	30	0	1	0	0	0	0	1	1	0	0	0	199	27	90	
		SMD	0.025	1.5	30	1	2	1	0	0	2	2	3	0	0	0	158	19	63	
		SMMD	0.1	1.7	30	0	0	0	0	2	1	1	3	0	0	0	213	23	77	

* SD = sulfadiazine, SMD = sulfamerazine, SMMD = sulfamethazine

† These doses were administered 2, 8, 14 and 22 hours after infection and at 8 hour intervals thereafter for four additional doses

‡ These values are the concentrations of drug occurring in the blood at the time midway between treatments, i.e., 4 hours after dosage

receiving the other drugs. These differences in activity were noted both when equal doses were administered and when equal blood levels were maintained.

Friedlander's bacillus infections. The results of the experiments with Friedlander's bacilli are summarized in table 3. As in the experiments with β hemolytic streptococci, the 3 drugs had essentially the same activity against one strain (E) and different degrees of activity against the other (GH). In the work with strain GH, sulfamerazine and sulfadiazine had essentially equal activities when

administered in equal doses; both drugs were much more effective than sulfamethazine. When equal blood levels of the 3 sulfonamides were maintained, sulfadiazine was considerably more effective than sulfamerazine, which in turn was significantly more active than sulfamethazine.

Staphylococcus aureus infections: The experiments with staphylococci have been summarized in table 4. In infections with both the Smith and 679 strain,

TABLE 3

Comparative effects of sulfadiazine, sulfamerazine and sulfamethazine on infections with Friedlander's bacillus in mice

STRAIN	NO. OF ORGANISMS IN INFECTING DOSE	DRUG*	DOSE† OF DRUG	AVER. CONC. OF DRUG IN BLOOD‡	NO OF MICE INFECTED	DEATHS ON DAY										AVER. HOURS SURVIVAL OF MICE THAT DIED	SURVIVORS	
						1	2	3	4	5	6	7-10	11-20	21-30	No.		%	
Equal doses of drug																		
GH (type A)	23,100	None	mgm.	mgm. %	20	19	1	0	0	0	0	0	0	0	15	0	0	
		SD	1.0	9.8	28	0	0	0	0	1	0	3	10	1	294	13	46	
		SMD	1.0	12.0	30	0	0	0	1	0	0	2	11	1	313	15	50	
		SMMD	1.0	7.4	29	1	0	3	15	4	2	3	1	0	103	0	0	
E (type B)	20,300	None			20	20	0	0	0	0	0	0	0	16	0	0		
		SD	0.5	5.8	30	0	1	0	0	0	0	12	7	0	241	10	33	
		SMD	0.5	7.8	30	0	1	0	0	0	0	12	5	1	245	11	36	
		SMMD	0.5	4.4	30	0	0	0	0	0	2	10	0	0	181	9	30	
Equal concentrations of drug in blood																		
GH (type A)	18,100	None			20	20	0	0	0	0	0	0	0	10	0	0		
		SD	0.5	5.8	30	3	0	0	0	0	0	8	3	0	174	16	53	
		SMD	0.5	7.8	30	5	2	1	0	0	0	9	5	0	157	8	27	
		SMMD	1.0	7.4	30	1	1	21	4	2	1	0	0	0	69	0	0	
E (type B)	18,400	None			20	20	0	0	0	0	0	0	0	12	0	0		
		SD	0.5	5.8	30	4	0	0	0	1	0	9	3	0	168	13	43	
		SMD	0.5	7.8	30	5	1	0	0	0	0	13	2	0	158	9	30	
		SMMD	1.0	7.4	30	1	0	0	0	0	0	21	1	0	196	7	23	

* SD = sulfadiazine; SMD = sulfamerazine; SMMD = sulfamethazine.

† These doses were administered 2, 8, 14 and 22 hours after infection and every 8 hours thereafter for five additional days.

‡ These values are the concentrations of drug occurring in the blood at the time midway between treatments, i.e., 4 hours after dosage.

sulfadiazine proved to be somewhat more effective than sulfamerazine or sulfamethazine. This was true when equal doses of the drugs were administered as well as when equal concentrations were maintained in the blood. There was no difference in the activity of equal doses of sulfamerazine and sulfamethazine, but when equal blood levels of these compounds were maintained, sulfamethazine was somewhat more active than sulfamerazine.

Dysentery bacillus infections. The results with *dysentery bacilli* are summarized in table 5. As in the experiments with *staphylococci*, there was a distinct dif

TABLE 4

Comparative effects of sulfadiazine, sulfamerazine and sulfamethazine on *Staphylococcus aureus* infections in mice

STRAIN	NO. OF ORGANISMS IN INJECT- ING DOSE	DRUG*	DOSE (mg)	AVER- AGE CONC. OF DRUG IN BLOOD†	NO. OF MICE IN- FECTED	DEATHS ON DAY										AVER- AGE PERCENT SURVIVAL OF MICE THAT DIED	SURVIVORS	
						1	2	3	4	5	6	7	8	9	10		No.	%
1 gual doses of drug																		
#679	73 000	None	mem	mem %	20	15	2	0	0	0	0	0	0	0	0	22	0	0
		SD	0.1	2.4	21	3	11	0	0	1	0	3	1	0	0	74	10	31
		SMD	0.1	3.4	30	21	7	0	0	0	0	0	0	0	0	21	2	6
		SMMD	0.1	1.7	30	19	9	0	0	0	0	0	0	0	0	26	2	6
Smith	65 500	None			20	20	0	0	0	0	0	0	0	0	0	13	0	0
		SD	0.1	2.4	30	22	1	0	1	1	1	1	0	0	0	37	3	10
		SMD	0.1	3.1	30	30	0	0	0	0	0	0	0	0	0	14	0	0
		SMMD	0.1	1.7	30	30	0	0	0	0	0	0	0	0	0	14	0	0
1 gual concentrations of drug in blood																		
#679	35 000	None			20	20	0	0	0	0	0	0	0	0	0	22	0	0
		SD	0.1	2.4	30	1	7	1	3	1	0	2	2	1	0	183	7	23
		SMD	0.05	2.2	30	25	7	0	0	0	0	0	0	0	0	23	0	0
		SMMD	0.2	2.6	30	7	14	0	1	1	1	1	1	0	0	56	4	13
Smith	10 100	None			20	20	0	0	0	0	0	0	0	0	0	13	0	0
		SD	0.2	3.6	30	0	0	0	1	0	0	0	3	0	0	216	26	87
		SMD	0.1	3.4	30	23	0	0	0	0	1	0	0	0	0	21	2	6
		SMMD	0.4	3.9	30	0	2	1	0	0	2	1	7	1	0	218	13	43
#679	114 500	None			20	16	4	0	0	0	0	0	0	0	0	23	0	0
		SD	0.2	3.6	30	0	2	0	0	0	1	2	3	0	0	176	22	73
		SMD	0.1	3.4	30	11	15	0	1	0	0	1	1	0	0	48	1	3
		SMMD	0.1	3.8	30	0	0	0	0	0	1	0	0	3	0	452	26	87
Smith	71 000	None			20	20	0	0	0	0	0	0	0	0	0	14	0	0
		SD	0.4	5.1	30	1	0	0	0	0	0	0	0	1	0	317	23	91
		SMD	0.2	5.0	30	2	7	0	0	1	0	2	2	2	0	209	18	60
		SMMD	0.8	6.1	30	0	0	0	0	0	0	0	1	2	0	526	27	90

* SD = sulfadiazine SMD = sulfamerazine SMMD = sulfamethazine

† These doses of drug were administered by stomach tube 2, 8, 14 and 22 hours after infection and every 8 hours thereafter for four additional doses

‡ These values are the concentrations of drug occurring in the blood at the time midway between treatments, i.e., 4 hours after dosage

ference in the activities of the 3 drugs. Against each of the strains and in both the dose for dose and blood level for blood level comparisons, sulfadiazine was

uniformly more effective than sulfamerazine or sulfamethazine. Dose for dose, sulfamerazine was slightly more active than sulfamethazine. However, when these two drugs were compared on the basis of equal blood levels, sulfamethazine was distinctly more active than sulfamerazine.

TABLE 5

Comparative effects of sulfadiazine, sulfamerazine and sulfamethazine on infections with dysentery bacilli in mice

STRAIN	NO. OF ORGANISMS IN INFECTING DOSE	DRUG*	DOSE OF DRUG†	AVER. CONC. OF DRUG IN BLOOD‡	NO. OF MICE INFECTED	DEATHS ON DAY										AVER. HOURS SURVIVAL OF MICE THAT DIED	SURVIVORS	
						1	2	3	4	5	6	7-10	11-20	21-30	No.		%	
Equal doses of drug																		
Bennett (<i>Shigella paradysenteriae</i> Flexner)	2,200	None			20	20	0	0	0	0	0	0	0	0	21	0	0	
		SD	0.005	0.4§	30	16	0	0	0	0	0	3	1	111	10	33		
		SMD	0.005	0.7§	29	24	0	0	0	0	0	1	0	38	4	17		
		SMMD	0.005	0.2§	29	27	1	0	0	0	0	1	0	31	0	0		
Cheatam (<i>Shigella sonnei</i>)	2,000	None			20	19	0	0	0	0	1	0	0	0	27	0	0	
		SD	0.1	2.4	30	1	3	0	0	0	1	0	1	183	23	77		
		SMD	0.1	3.4	30	6	10	0	0	0	1	0	0	42	12	40		
		SMMD	0.1	1.7	30	12	15	0	0	0	0	0	0	26	3	10		
Equal concentrations of drug in blood																		
Bennett (<i>Shigella paradysenteriae</i> Flexner)	1,400	None			20	16	4	0	0	0	0	0	0	0	21	0	0	
		SD	0.01	0.6§	30	5	3	1	0	0	0	1	1	1	114	18	60	
		SMD	0.005	0.7§	30	17	6	0	1	0	1	2	0	53	3	10		
		SMMD	0.025	0.6§	30	8	7	1	1	1	0	2	0	0	53	7	23	
Cheatam (<i>Shigella sonnei</i>)	240	None			20	20	0	0	0	0	0	0	0	0	17	0	0	
		SD	0.1	2.4	30	0	1	0	0	0	0	3	2	0	214	24	80	
		SMD	0.05	2.2	30	9	12	1	2	0	0	0	1	1	67	4	13	
		SMMD	0.2	2.6	30	1	12	1	0	0	0	2	3	0	100	11	37	

* SD = sulfadiazine; SMD = sulfamerazine; SMMD = sulfamethazine.

† These doses of drug were administered 2, 8, 14 and 22 hours after infection and every 8 hours thereafter for four additional doses.

‡ These values are the concentrations of drug in the blood at the time midway between treatments, i.e., 4 hours after dosage.

§ These values are estimates taken from the graphs shown in figure 1, since the methods used for determining the concentrations of drug in the blood are not sensitive enough to determine such small amounts accurately.

Pneumococcal meningitis in rats. The activities of sulfathiazole, sulfadiazine, sulfamerazine, and sulfamethazine against pneumococcal meningitis in rats are summarized in table 6. According to the numbers of rats surviving when either 20 or 40 mgm. doses of these drugs were administered, sulfamerazine seemed to be the most effective derivative and sulfathiazole the least effective. On the 20

mgm dosage, sulfadiazine and sulfamethazine were somewhat less effective than sulfamerazine. On the 40 mgm dosage, however, sulfamethazine was definitely more effective than sulfadiazine and was essentially as active as sulfamerazine.

As shown in the table, many of the rats that survived on treatment with the various drugs had residual symptoms of meningeal infection. These symptoms consisted of inability to coordinate movements, neck rigidity and hyperirritability. It is interesting to note that comparatively few of the rats treated with sulfathiazole showed these residual symptoms, and furthermore that the numbers of completely recovered rats (i.e., the number of survivors less the number with

TABLE 6

*Comparative effects of sulfathiazole, sulfadiazine, sulfamerazine and sulfamethazine on pneumococcal meningitis in rats**

DRUG†	DOSE OF DRUG†	CONC. OF DRUG IN BLOOD‡	NO. OF RATS INFECTED	DEATHS ON DAY										AVER. HOURS SURVIVAL OF RATS THAT DIED	SURVIVORS		RECOVERED RATS WITH RESIDUAL SYMPTOMS	
				1	2	3	4	5	6	7	8	9	10		No.	%	No.	%
None	0	0	37	2	2	16	10	4	1	0	1	0	1	88	1	3	0	0
ST	20	0.9	40	0	1	3	8	6	7	4	0	0	0	123	11	27	2	18
SD	20	7.16	40	0	0	1	0	0	9	7	1	0	0	166	22	55	13	59
SMD	20	15-31	10	0	0	1	0	0	0	12	0	0	0	184	27	63	15	55
SMMD	20	8-26	40	0	0	1	1	0	5	15	1	0	0	177	17	42	6	35
ST	40	1.15	37	0	0	0	2	3	9	5	0	0	0	148	18	49	2	11
SD	40	14.26	39	0	0	0	0	2	0	11	0	0	0	191	26	67	14	54
SMD	10	25.53	39	0	0	0	0	1	0	3	0	0	0	189	36	90	18	50
SMMD	40	14.39	39	0	0	0	0	0	0	1	1	0	0	219	34	87	20	59

* Infecting organism, type I pneumococcus, strain McGovern, infecting dose approximately 6000 organisms.

† ST = sulfathiazole, SD = sulfadiazine, SMD = sulfamerazine, SMMD = sulfamethazine.

‡ These doses of drug were administered by stomach tube, 2 and 8 hours after infection and at 8 a.m. and 4 p.m. for 4 additional days.

§ These values give the daily high and low concentrations of drug in the blood.

symptoms) were essentially the same in each of the groups treated with the 4 different drugs.

Attention should be called to the fact that in the above experiments there were considerable differences in the concentrations of the different sulfonamides in the blood. The levels of sulfathiazole were much lower than those of sulfadiazine and sulfamethazine, which in turn were somewhat lower than those of sulfamerazine. The activity of sulfathiazole was surprisingly high in view of the low blood level, and it might be predicted that its activity would have been equal or superior to that of sulfamerazine if the same concentration had been maintained in the blood.

In vitro activity

METHODS.

The *in vitro* activities of sulfamerazine and sulfamethazine were compared with those of sulfadiazine and sulfathiazole against pneumococci, staphylococci, Friedlander's bacilli, dysentery bacilli and *E. coli*. All strains used in the *in vivo* experiments were tested *in vitro*. In addition 3 other strains of pneumococci and 2 strains of *E. coli* were studied.

Basal media: Since there are indications that the composition of the test medium affects both the absolute and relative *in vitro* activities of different sulfonamides (17-19), the tests in this study have been carried out in both a simple and a complex medium whenever possible. The following basal media were used: (a) *Beef heart broth*. This was an infusion broth (prepared from Difco dehydrated beef heart) to which was added 2% neopeptone and 0.5% sodium chloride. The pH of this medium was 7.8. The medium was used without enrichment in the tests with staphylococci, Friedlander's bacilli, dysentery bacilli and *E. coli*. In the experiments with pneumococci it was necessary to enrich the medium with 2% defibrinated rabbit blood in order to obtain consistent growth. (b) *Synthetic medium*—This was the medium described by Sahyun and coworkers (20) enriched with 0.1% casein hydrolysate (Smao Vitamin Free). This medium was used in experiments with Friedlander's bacilli, dysentery bacilli and *E. coli*.

Preparation of sulfonamide-containing media: Concentrated solutions of the various sulfonamides were prepared in the basal media, diluted and tubed in 9 cc. quantities. After all constituents, including inoculum and enrichment, were added, the sulfonamide concentrations in the beef heart medium were 0, 0.3, 0.6, 1.25, 2.5, 5, 10, 20, 40, 80 and 160 mgm. %. The final concentrations in the synthetic medium were 0, 0.02, 0.04, 0.08, 0.16, 0.3, 0.6, 1.25, 2.5 and 5.0 mgm. %.

Test procedure: The pneumococci, staphylococci and Friedlander's bacilli were obtained from a mouse passage culture. The dysentery bacilli and *E. coli* were obtained from stock cultures. These organisms were subcultured twice in the medium in which the test was to be performed; each subculture was incubated 12 hours. The second subculture was diluted serially in the appropriate basal medium and 1 cc. quantities of a 10^{-6} dilution were added to each of a series of tubes containing the various concentrations of the respective sulfonamides; this gave an inoculum of from 150 to 600 organisms per cc. The resulting cultures were incubated at 37.5° for 48 hours and visual estimations of growth were made at the end of 12, 24 and 48 hours incubation. In the tests with the synthetic and plain beef heart media, turbidity was used as the criterion of growth. In the tests with beef heart medium enriched with blood, both turbidity and the change in color of the red cell sediment were noted. Only results obtained after 24 hours incubation are recorded below; these observations did not differ significantly from those at 48 hours.

At least one duplicate test of activity, and in most instances two were carried out with each organism. The data presented in tables 7 and 8 are representative of all tests.

RESULTS. *Pneumococci:* The data in table 7 show that sulfamethazine was slightly more effective than sulfamerazine in inhibiting the *in vitro* growth of pneumococci; sulfamerazine in turn was just slightly more effective than sulfadiazine. Sulfathiazole was considerably more active than any of the three diazines. The differences in activity of sulfamethazine, sulfamerazine and sulfadiazine were not great, nor were they evident with all the strains of pneumococci. The differences did occur regularly in repeat tests with certain strains, however; this would seem to justify the conclusion that against some pneumococci, sulfamethazine and sulfamerazine have slightly greater activity than sulfadiazine.

Staphylococci: As shown in table 8, sulfamethazine was somewhat more effective than either sulfamerazine or sulfadiazine in inhibiting the growth of staphy-

lococci Against one of the two strains (Smith), the activity of sulfamethazine was equal to that of sulfathiazole, against the other strain (679) sulfamethazine was less effective. The activity of sulfamerazine was identical with that of sulfadiazine.

TABLE 7

Activities of sulfathiazole, sulfadiazine, sulfamerazine and sulfamethazine against pneumococci in vitro

Test in beef heart medium enriched with rabbit blood

STRAIN OF PNEUMOCOCCI	MAXIMUM SULFONAMIDE* CONCENTRATION PERMITTING VISIBLE GROWTH (MG% PER CENT)			
	ST	SD	SMD	SMMD
Type I McGovern	1.25	10.0	10.0	5.0
Type I SN 1	0.6	5.0	2.5	1.25
Type II CH	0.6	5.0	2.5	1.25
Type III CHA	1.25	5.0	5.0	2.5
Type III Wistuba	2.5	10.0	5.0	5.0

* ST = sulfathiazole, SD = sulfadiazine, SMD = sulfamerazine, SMMD = sulfamethazine

TABLE 8

Activities of sulfathiazole, sulfadiazine, sulfamerazine and sulfamethazine against Friedlander's bacilli, staphylococci, dysentery bacilli and *E. coli*, in vitro

ORGANISM AND STRAIN	MAXIMUM SULFONAMIDE* CONCENTRATION PERMITTING VISIBLE GROWTH (MG% PER CENT)							
	Test in beef heart medium				Test in synthetic medium			
	ST	SD	SMD	SMMD	ST	SD	SMD	SMMD
Friedlander's bacillus								
GH—Type A	40	160	160	160	0.08	0.01	0.08	0.32
E—Type B	20	80	80	80	0.16	0.16	0.16	0.16
<i>Staphylococcus aureus</i>								
#679	10	80	80	40				
Smith	10	40	40	10				
Dysentery bacillus								
Bennett	10	40	40	40	0.02	0.01	0.01	0.16
Cheatam	20	80	80	80	0.08	0.16	0.16	0.32
<i>E. coli</i>								
CH	80	160	160	160	0.08	0.08	0.08	0.32
MacLeod	40	160	160	160	0.16	0.16	0.16	0.64

* ST = sulfathiazole, SD = sulfadiazine, SMD = sulfamerazine, SMMD = sulfamethazine

Friedlander's bacilli The activities of sulfamerazine, sulfamethazine and sulfadiazine against strain E were essentially identical, both in the simple and complex media. In the complex medium these sulfonamides were less effective than sulfathiazole, but in the synthetic medium they were equally active. In the tests

with strain GH in the simple medium, sulfamethazine was distinctly less effective than the other drugs; sulfamerazine and sulfathiazole were slightly less active than sulfadiazine. Since strain GH grew in the beef heart medium in the highest available concentrations of sulfamerazine, sulfamethazine or sulfadiazine, the tests in this medium were inconclusive, except for showing that sulfathiazole was more active than any of the diazines.

Dysentery bacilli: In the tests in beef heart medium, sulfamerazine, sulfamethazine and sulfadiazine had essentially the same activity, each drug being approximately one-fourth as active as sulfathiazole. In the tests in synthetic medium, however, sulfamethazine had less activity than sulfamerazine and sulfadiazine, which were about equally active; sulfathiazole was the most active of the 4 drugs.

TABLE 9

Summary of in vivo activity of sulfamerazine and sulfamethazine as compared with sulfadiazine

ORGANISM	STRAIN	RELATIVE ACTIVITY OF DRUGS*	
		On equal doses	On equal blood levels
Pneumococcus	McGovern SV-1	SMD > SMMD > SD SMD > SMMD > SD	SMMD = SMD > SD
β hemolytic streptococcus	C203 CF1	SD = SMMD = SMD SD > SMMD > SMD	SD = SMMD = SMD SD > SMMD > SMD
Friedlander's bacillus	GH E	SD = SMD > SMMD SD = SMD = SMMD	SD > SMD > SMMD SD = SMD = SMMD
Staphylococcus aureus	679 Smith	SD > SMD = SMMD SD > SMD = SMMD	SD > SMMD > SMD SD > SMMD > SMD
Dysentery bacil- lus	Bennett Cheatam	SD > SMD > SMMD SD > SMD > SMMD	SD > SMMD > SMD SD > SMMD > SMD

* SD = sulfadiazine; SMD = sulfamerazine; SMMD = sulfamethazine.

E. coli: In the beef heart medium, the tests on the activity of the diazines against *E. coli* gave inconclusive results, growth occurring in the topmost concentrations available; these tests merely showed that sulfathiazole had greater activity than the other drugs. In the synthetic medium, however, sulfamethazine was again only one-fourth as active as sulfamerazine and sulfadiazine. These latter drugs were as effective as sulfathiazole.

DISCUSSION. The essential features of the *in vivo* study on the relative chemotherapeutic activities of sulfamerazine, sulfamethazine, and sulfadiazine, have been summarized in table 9. Particular attention should be paid to the differences in the effectiveness of the 3 sulfonamides when equal concentrations were present in the blood. These data, which give a better indication of comparative activity than the equal dose results, permit two conclusions. First, the relative chemotherapeutic activities of sulfamerazine, sulfamethazine, and

sulfadiazine depend to a large extent on the species of bacteria used as the infecting agent. Secondly, sulfadiazine, although not in every case the most effective compound, has somewhat broader activity than the other drugs.

In infections with pneumococci, both in the mouse and in the rat, sulfamerazine and sulfamethazine were slightly but definitely superior to sulfadiazine. However, against infections with β hemolytic streptococci and Friedlander's bacilli, the 3 drugs were either equally active or sulfadiazine was the most effective. Against infections with staphylococci and dysentery bacilli, sulfadiazine was clearly the most active of the 3 drugs.

The derivatives, sulfamerazine and sulfamethazine, had nearly identical activity against infections with pneumococci. In Friedlander's bacillus infections, sulfamerazine was slightly more effective than sulfamethazine. However, sulfamethazine was consistently more effective than sulfamerazine in infections with β hemolytic streptococci, staphylococci, and dysentery bacilli. It is noteworthy that in pneumococcal and Friedlander's bacillus infections, which require long term treatment, sulfamerazine was as effective as or even more effective than sulfamethazine. However, in infections requiring shorter term treatment, i.e. those with β hemolytic streptococci, staphylococci, and dysentery bacilli, sulfamethazine was the more active drug. In this connection, it should be pointed out that the absorption and excretion characteristics of the two drugs differ considerably (1, 21, 22). Although in the above experiments the mean levels of these sulfonamides were essentially the same, the peak levels of sulfamethazine were greater than those of sulfamerazine, whereas the low levels of the latter drug were higher than those of sulfamethazine. The sustained levels of sulfamerazine may account for the greater effectiveness of this drug against infections requiring extended treatment, whereas the high peak levels of sulfamethazine may explain its effectiveness in infections requiring only short term treatment.

Assuming that the experimental data may give an indication of the clinical effectiveness of these drugs, the question may be raised as to which of the compounds is the most desirable sulfonamide. Since sulfamerazine and sulfamethazine are not as effective against as wide a variety of organisms as is sulfadiazine, the latter compound may seem to be the superior drug. This assumes that the selection of a sulfonamide is based chiefly on its chemotherapeutic activity. Such is the case when dealing with compounds of widely differing activities. It should be pointed out, however, that the drugs used in this study all possessed comparatively high chemotherapeutic activity. Even though there were differences in their activities, these were small as compared with differences existing among other commonly used sulfonamides.

In view of the fact that all three drugs possessed considerable therapeutic activity, other factors may be considered in deciding their suitability for clinical use. The ease of maintaining therapeutically effective blood levels and the toxicity of the sulfonamide for the host are also important factors. Previous studies (1, 21, 22) have shown that sulfamerazine is superior to sulfadiazine both in respect to the ease of maintaining effective blood levels and in respect to renal

toxicity. Sulfamethazine has considerably less renal toxicity than either sulfamerazine or sulfadiazine (1) but is absorbed and excreted so rapidly as to make it difficult to maintain effective blood levels (1, 21, 22). Considering all these characteristics there would seem to be little choice between sulfamerazine, sulfamethazine and sulfadiazine. In individual cases where there is need for a particular property, one of these drugs may be better than the others. Thus if used in a patient with proved renal dysfunction, sulfamethazine with its low renal toxicity might be the drug of choice. If conditions made necessary comparatively infrequent medication and yet required well sustained blood levels, sulfamerazine would be the more desirable drug. In very severe infections, where use of the most active drug would be imperative, sulfadiazine, in most instances, would be the drug of choice.

The results of the *in vitro* tests permit few conclusions and have not been considered in the above evaluation. With the exception of the experiments with pneumococci, the data are not in accord with those obtained *in vivo*. Such discrepancies have been noted frequently in the past (19, 23-25). They most likely arise from the fact that the mouse presents a different environment to the organism than an artificial medium. Since the relative and absolute activities of the sulfonamides depend on the composition of the medium, it is not surprising that results of *in vivo* and *in vitro* tests should differ.

Another observation made in this study deserves mention, even though it does not bear on the relative activities of the 3 sulfonamides. The absolute amounts of any of these drugs required to protect 50% of the mice varied enormously with different infecting agents. With sulfadiazine for example, much larger doses were required to protect mice against pneumococci and Friedlander's bacilli than were required in infections with β hemolytic streptococci, staphylococci and dysentery bacilli. These findings might suggest that there are also differences in the amounts of sulfonamide needed for treatment of various human infections. Clinical investigation of this point might lead to lower dosage against certain infections and coincidentally reduce the numbers of toxic reactions associated with sulfonamide therapy.

SUMMARY

The comparative activities of sulfamerazine, sulfamethazine, and sulfadiazine against experimental infections with pneumococci, β hemolytic streptococci, Friedlander's bacilli, staphylococci and dysentery bacilli have been determined. The results have shown that sulfamerazine and sulfamethazine are slightly superior to sulfadiazine in the treatment of pneumococcal septicemia in mice and meningitis in rats. Sulfadiazine was as effective as or slightly more effective than the other drugs against infections with β hemolytic streptococci and Friedlander's bacilli and was distinctly superior against infections with staphylococci and dysentery bacilli. The bearing of these observations on the clinical use of the 3 drugs has been discussed.

In vitro experiments have also been carried out to determine the capacities of sulfamerazine, sulfamethazine and sulfadiazine to inhibit the growth of pneumococci, Friedlander's bacilli, staphylococci, dysentery bacilli and *E. coli*. As

was noted previously, the relative activities of the drugs depended upon the test medium. In the complex medium, sulfamerazine and sulfamethazine were somewhat more active than sulfadiazine against pneumococci, whereas sulfamethazine was the most active drug against staphylococci, the 3 drugs had essentially the same activity against the other organisms. In the synthetic medium, sulfamerazine and sulfadiazine were equally active against Friedländer's bacilli, *E. coli* and dysentery bacilli and were considerably more active than sulfamethazine. These results did not agree with the *in vivo* findings. An explanation for the discrepancy has been attempted.

REFERENCES

- (1) SCHMIDT, L. H., H. B. HOORNS, E. A. BADGER AND I. C. SCHMIDT, THIS JOURNAL, 81, 17, 1944
- (2) ROBLIN, R. O., JR., J. H. WILLIAMS, P. S. WINNEK AND J. P. ENGLISH, J. Amer. Chem. Soc., 62, 2002, 1940
- (3) CALOWELL, W. T., E. C. KORNFELD AND C. K. DONNELL, J. Amer. Chem. Soc., 63, 2183, 1941
- (4) SPRADOE, J. M., L. W. KISSINOFF AND R. M. LINCOLN, J. Amer. Chem. Soc., 63, 3028, 1941
- (5) FELLOWY, E. J., Proc. Exper. Biol. and Med., 48, 680, 1941
- (6) ROBLIN, R. O., JR., P. S. WINNEK AND J. P. ENGLISH, J. Amer. Chem. Soc., 64, 567, 1942
- (7) MARSHALL, E. K., JR., J. T. LITCHFIELD, H. J. WHITE, A. C. BRATTON AND K. C. SHEPHERD, THIS JOURNAL, 76, 226, 1942
- (8) ROSE, F. L., A. R. MARTIN AND H. G. J. BEVAN, THIS JOURNAL, 77, 127, 1943
- (9) FINSTON, W. H., R. H. FOLLIS AND R. D. WILLIAMS. Unpublished observations
- (10) MACARTNEY, D. W., G. S. SMITH, R. W. JUXTON, W. A. RAMSAY AND J. COIDMAN, Lancet, I, 639, 1942
- (11) CEFTER, W. I., S. B. ROSE, A. H. DOWN AND H. I. FLIPPIN, Amer. J. Med. Sci., 206, 211, 1943
- (12) FLIPPIN, H. F., W. I. CEFTER, A. H. DOWN AND J. H. CLARK, Amer. J. Med. Sci., 206, 216, 1943
- (13) HALL, W. H., AND W. W. SPINK, J. A. M. A., 123, 125, 1943
- (14) LIPPER, M. H., L. K. SWEET AND H. F. DOWLING, J. A. M. A., 123, 134, 1943
- (15) HAGEMAN, P. O., C. C. HARFORD, S. S. SOBIN AND R. E. AHRENS, J. A. M. A., 123, 325, 1943
- (16) COOPER, M., AND H. M. KELLER, Personal communication
- (17) WHITE, H. J., J. T. LITCHFIELD, JR., AND E. K. MARSHALL, JR., THIS JOURNAL, 73, 104, 1941
- (18) STRAUSS, L., AND M. FINLANO, Proc. Soc. Exper. Biol. and Med., 47, 428, 1941
- (19) SESLER, C. L., AND L. H. SCHMIDT, THIS JOURNAL, 79, 117, 1943
- (20) SAHYUN, M., P. BEARD, E. W. SCHULTZ, J. SNOW AND F. CROSS, J. Infect. Dis., 58, 28, 1936
- (21) GOODWIN, R. A., JR., O. L. PETERSON AND M. FINLANO, Proc. Soc. Exper. Biol. and Med., 51, 262, 1942
- (22) WELCH, A. D., P. A. MATTIS, A. R. LATVEN, W. M. BENSON AND E. H. SHIELDS, THIS JOURNAL, 77, 357, 1943
- (23) SCHMIDT, L. H., C. HILLES, H. A. DETTWILER AND E. STARKS, J. Infect. Dis., 67, 232, 1940
- (24) WHITE, H. J., A. C. BRATTON, J. T. LITCHFIELD, JR. AND E. K. MARSHALL, JR., THIS JOURNAL, 72, 112, 1941
- (25) SCHMIDT, L. H., J. M. RUFSCORGE, C. L. SESLER AND M. HAMBURGER, JR., THIS JOURNAL, 73, 465, 1941

THE EFFECTS OF SULFANILAMIDE AND AZIDE ON OXYGEN CONSUMPTION AND CELL DIVISION IN THE EGG OF THE SEA URCHIN, *ARBACIA PUNCTULATA*

KENNETH C. FISHER, R. J. HENRY AND E. LOW

Marine Biological Laboratory, Woods Hole, Mass.

Received for publication January 4, 1944

INTRODUCTION. Experiments with narcotics have led to the conclusion that the chain of respiratory reactions upon which cell division depends in yeast (Fisher and Stern, '42), the sea urchin egg (Fisher and Henry, '44) and a ciliate (Ormsbee and Fisher, '44) is responsible for only a fraction of the total oxygen consumption of these cells. This conclusion is of interest in connection with the chemotherapeutic properties of the sulfonamide drugs for, in the first instance, these substances act to slow or stop cell division. If, indeed, growth does depend upon a specific system of respiratory reactions, the "activity" system, then it is evident that the action of the abiotics may be exerted on that system (Fisher, '42). The testing of this possibility involves the technical difficulties common to investigations on the mechanism of sulfonamide effects on one hand and to all research on growing cells on the other, difficulties which have not always been properly appreciated (Henry, '44). It is necessary, for example, to conduct the experiments in such a way that the effects of the composition of the medium are taken into account; and where rates of reactions are involved, the amount of living material present upon which must rest any statement of rates, either must not change or else the nature and magnitude of the change must be known. There are several approaches to the general problem which minimize or completely remove these difficulties. One of the most obvious involves the use of cells which are relatively independent of the external environment. It has long been appreciated that the egg of the sea urchin is such a cell and it has already been reported that sulfanilamide inhibits cell division in it (Thomas, '41). Our investigation of the mechanism of the action of the sulfonamides has therefore begun with a number of observations on these cells. The data reported herewith show the effects of sulfanilamide, azide, urethane and combinations of these inhibitors on both cell division and oxygen consumption. They lead to the conclusion that when cell division is stopped by a sulfonamide the activity system is also inhibited completely.

MATERIAL AND METHODS. Fertilized (dividing) and unfertilized (resting) eggs of the sea urchin *Arbacia punctulata* were used in this investigation. The methods employed in obtaining the eggs, and in determining the rate of oxygen consumption and of cell division have already been described (5). In the respiration experiments the various inhibiting agents were in every case added directly to the eggs at the time the respirometer vessels were filled. As in the preceding research (5) the temperature was maintained at 25°C.

RESULTS. *Sulfanilamide on the fertilized egg.* In fifteen experiments the effects of various concentrations of sulfanilamide on the oxygen consumption

of fertilized eggs was determined. These data together with the results of ten experiments on the rate of cell division are given in figure 1. Sulfanilamide slows the rate of division and this inhibition is accompanied by a depression of the rate of oxygen consumption. Complete suppression of cell division is associated with a reduction of the respiratory rate to approximately 55% of the normal value. The limited solubility of sulfanilamide in sea water prevented the use of solutions more concentrated than 0.04M. The relation between concentration and effect is, consequently, not defined completely.

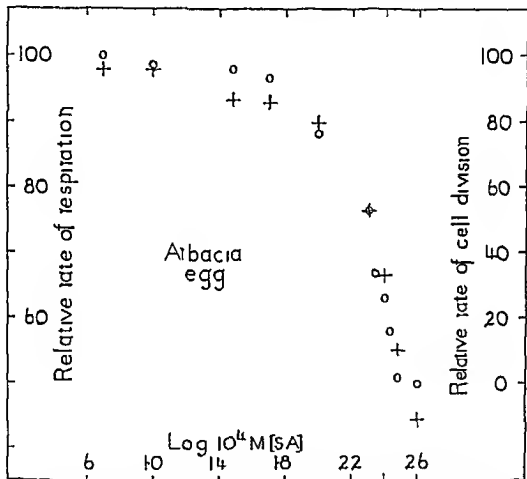


FIG. 1. THE RATE OF OXYGEN CONSUMPTION (CROSSES) AND RATE OF CELL DIVISION (CIRCLES) IN THE SEA URCHIN EGG AT DIFFERENT CONCENTRATIONS OF SULFANILAMIDE (SA).

On the average each point for respiration is the mean of 5 separate determinations while for cell division each is the mean of 4 determinations.

enough to warrant the type of analysis used for narcotics (5). The effect of 0.04M sulfanilamide on cell division was found to be completely reversible.

Experiments on cell division were also made using sulfapyridine, sulfathiazole and sulfadiazine. Though all three inhibited, it was not possible to bring about a complete block of division with either of the first two substances mentioned because of their low solubility. Sulfadiazine lowered the pH of the sea water and it is very probable that a large part of its effect was due to the pH change rather than to sulfadiazine itself. When combinations of sulfanilamide, sulfapyridine,

and sulfathiazole were used, a partial summation of their independent effects was observed.

As indicated in experiments on cell division p-amino benzoic acid in concentrations ranging from $1 \times 10^{-7}M$ to $2.6 \times 10^{-3}M$ did not exhibit any anti-sulfonamide effect.

Sulfanilamide and azide on the unfertilized egg. Five experiments were made to test the effect of sulfanilamide on the consumption of oxygen by the resting cells. The most concentrated solution used, 0.04M, removed only 5-10% of the oxygen consumption. Less concentrated solutions not only did not inhibit but, on the contrary, produced a slight (10-15%) acceleration of the respiration.

In six separate experiments the effects of five different concentrations of sodium azide were determined. Expressed as a per cent of the rate observed in the absence of inhibitor the average respiration in each of the following molar concentrations of azide, .003, .0045, .007, .01, and .02, was found to be 92, 98, 87, 96 and 97, respectively. It will be noted that the slight inhibition which is suggested here does not vary consistently with the inhibitor concentration. Actually, the degree of inhibition is so slight as to approach the limit of reproducibility of the technique. It is concluded, therefore, that at least in this range of concentrations azide has no effect on the oxygen consumption of the unfertilized egg.

Krahl, Keltch, Neubeck and Clowes ('41) have recorded observations which show that azide produces a maximal inhibition of oxygen consumption in the fertilized egg amounting to approximately 50%. The concentration of azide necessary to stop cell division completely, approximately .01M, just produces the maximum inhibition of respiration. We have confirmed these observations.

Thus both azide and sulfanilamide, though inhibiting cell division and oxygen consumption in the fertilized egg, have little or no inhibiting effect in the unfertilized egg. It is difficult to escape the conclusion that fertilization introduces a respiratory system upon which cell division depends and which is responsible for 40-50% of the oxygen consumption of the fertilized egg (however c.f. Ball, '42). This system differs from the one which operates in the unfertilized egg, and which is presumably responsible for the remaining 50% of the respiration of the fertilized egg, by being sensitive to both azide and sulfanilamide. Such a conclusion is logical moreover, for the fertilized egg divides while the unfertilized egg does not, indicating that there are certainly some reactions in the fertilized which are not functional in the unfertilized egg.

Sulfanilamide in the presence of azide. It is evident that, if the respiration inhibited by sulfanilamide and azide is mediated by the same system of reactions, then, when a maximum inhibition by azide exists, there should be no further inhibition produced upon the addition of the sulfonamide. Table 1 shows the gradual increase of inhibition as the sulfanilamide concentration rises and that the maximum inhibition by azide is probably reached at a concentration of .009M. The addition of sulfanilamide to a preparation maximally inhibited with azide does not result in any appreciable increase in inhibition. It will be recalled that the maximum effect of azide is to reduce the respiration to ap-

proximately 50% of the normal, and that a similar inhibition is produced by the highest concentration of sulfanilamide employed in table 1. These observations along with the combination experiment just described make it evident that the two inhibitors do remove the same respiration.

The effect of urethane on the fertilized egg in the presence of azide. It is simplest to conclude from the experiments recorded above that at fertilization a series of chemical changes is initiated upon which cell division depends and which constitute approximately half of the normal respiration of the dividing cells. This conclusion is now independently indicated by (1) experiments with narcotics, (2) experiments with azide and (3) experiments with sulfanilamide. The data from (2) and (3) have been checked against one another using combinations of azide and sulfanilamide. A similar check involving the narcotic experiments is possible and has been made. It is somewhat more complicated because narcotics inhibit the basal system which is apparently common to both fertilized and unfertilized eggs as well as the activity system of reactions which is a unique characteristic of the fertilized egg. It now seems likely however that azide

TABLE 1

The relative rate of oxygen consumption by fertilized sea urchin eggs in different concentrations of sulfanilamide or azide and in combinations of these

The values are the average of three identical experiments

SULFANILAMIDE	AZIDE			
	0	0065M	009M	017M
0	100	63	56	55
0175M	82		42	
025M	64		48	
0375M	43		54	

inhibits only the activity system. With the maximum azide effect established then only the basal system should remain for inhibition by narcotics. It follows that the additional inhibition produced by urethane in the presence of 01M azide should exhibit the quantitative characteristics of the inhibition of the basal system or of the unfertilized eggs. The average data from six separate experiments, in which the inhibition by urethane of the azide stable oxygen consumption of the fertilized egg was examined are given in figure 2A. The respiration at different concentrations of urethane is expressed as a per cent of the value observed in 01M azide alone. The smooth curve is a good representation of all the points except the one at the highest concentration, i.e., 0.9M. The latter point was disregarded since concentrations of that order produce an irreversible damage relatively rapidly (5) implying that they exert a general harmful effect which is quite apart from the more specific reversible narcotic action seen with the lower concentrations. These data, as is indicated by the curve, suggest that the maximum effect of urethane is to lower the rate of respiration to 54% of the value in azide alone. In other words 54% of the azide stable respiration is also urethane stable.

Granting the estimation of the urethane-stable fraction and taking it into account, the data have been replotted in figure 2B on the double log axes most conveniently used to test the application of the law of mass action. The observations clearly conform now to a single straight line. In the presence of .01M azide then, it appears that the action of urethane may be exerted at only a single site in the cell and that it may be described by an expression of the mass law. When urethane is employed on the fertilized egg in the absence of azide, the evidence (5) points towards two sites for its action.

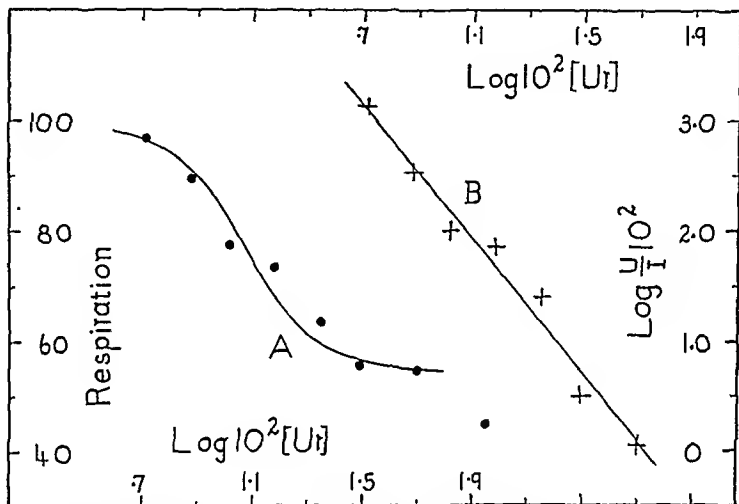


FIG. 2. THE RATE OF OXYGEN CONSUMPTION BY THE FERTILIZED EGG IN .01M AZIDE AS A FUNCTION OF THE URETHANE CONCENTRATIONS

Each point is on the average the mean of 5 determinations. In A (dots) the observed respiration in .01 M azide is plotted as a function of the logarithm of the urethane concentration. In B (crosses) the same data have been plotted to test the possibility of describing the effect of urethane in terms of the mass law. U is the number obtained by subtracting 54 (the urethane-stable %) from the respiration observed in azide plus urethane expressed as a % of the respiration in azide alone. I is the difference between the respiration in azide alone and the respiration observed in azide plus urethane, expressed as a % of the respiration in azide alone.

It may be argued that in the presence of azide the affinities of the two sites become similar so that even though two are present the combination results in a single line on the mass law plot. The alternative conclusion is that azide has removed one of the two sites normally present. The slope of the line in figure 2B giving the value of a in the expression of the mass law which describes the line, is 3.1. For comparison the equivalent data for the effect of urethane in the absence of azide, as determined in the previous research, are given in table 2. It is at once evident that the values for the fertilized egg in the presence of azide, for the unfertilized egg, and for the basal system of the fertilized egg, are all very similar. In all probability the same system of respiratory reactions is

being inhibited in all of these cases. The conclusion that the maximum effect of azide results in the complete removal of the activity system is inevitable.

Azide brings about approximately a 50% inhibition of the oxygen consumption of the fertilized egg. Fifty five per cent of the azide stable respiration is also urethane stable so that of the total oxygen consumption of the fertilized egg some 27.5% is urethane stable. This fact was not appreciated from the data obtained in the absence of azide. Reference to those data (5) however indicates that at the highest concentration of urethane the calculated line actually would

TABLE 2
Comparison of the values of a for urethane

	ACTIVITY SYSTEM	BASAL SYSTEM
Unfertilized eggs		4.0
Fertilized eggs	0.5	3.0
Fertilized eggs in 0.01M azide		3.1

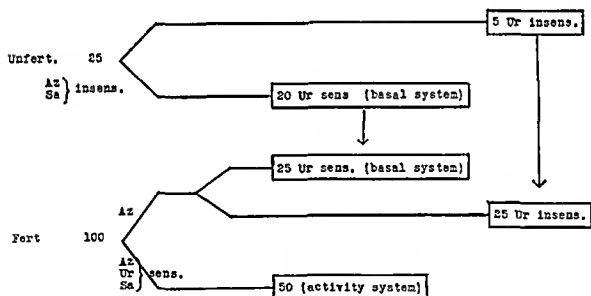


FIG. 3
sumption of
present and
approximate
indicated by
azide and sulfanilamide respectively

be a better representation of the observations if 25% of the total respiration were considered to be urethane stable. There is then no discrepancy between the two sets of experiments—the apparent difference arising from the greater sensitivity of the approach in the new observations.

It is appropriate at this point to summarize those metabolic changes in the sea urchin egg upon fertilization which are recorded here and in the previous paper. This is done in the following diagram (figure 3), in the preparation of which it was concluded from Korr's (37) work that the relative rates of oxygen

consumption in the fertilized and unfertilized eggs are respectively 100 and 25. The quantities shown in this schema are relative and, of course, approximate, but it would appear that the oxygen consumption contributed by the basal system is scarcely changed by fertilization (from 20 to 25). However, in addition to starting the activity system, the initiation of development by fertilization markedly increases the urethane insensitive respiration (from 5 to 25).

It is clear that the principal observations recorded in the present paper are most easily satisfied by the conclusion drawn from the original work with narcotics, namely that at fertilization there is initiated a series of respiratory reactions, the activity system, upon which division depends. The activity system accompanies in the fertilized egg a second chain of reactions, the basal system, which appears to be functional in the unfertilized egg as well. Being independent these two systems react independently with narcotics, with azide, and with sulfanilamide, thus giving rise to the various observations already noted. The data indicate that the effect of sulfanilamide is exerted on the activity system and this is undoubtedly the reason why sulfanilamide interferes with cell division.

DISCUSSION. Current interest in the sulfonamides arises almost entirely from the bacteriostatic and bactericidal properties of these compounds. It is appropriate to consider the significance of the observations on the sea urchin egg to the mechanism of the effects of sulfonamides in bacteria. The existence in a dividing cell of a discrete narcotic sensitive system of respiratory reactions which are closely related to cell division is not peculiar to the sea urchin egg. A similar situation exists in yeast and in a ciliate so that it may not be unreasonable to consider that it occurs generally and probably therefore in bacteria. It is quite definitely indicated that sulfanilamide in the sea urchin egg acts by blocking the activity system. The possibility must be entertained that in bacteria the mechanism of its action is the same.

It is important to appreciate that the lack of anti-sulfonamide effect by p-amino benzoic acid in the egg is not evidence that the mode of action in the two cases differ. Strictly all this discrepancy actually proves is that the enzymes concerned are not completely identical. It is readily conceivable that p-amino benzoic acid may be a "promoter" for the species of catalyst which is inhibited by the sulfonamides in bacteria without it necessarily having the same activity on the equivalent catalysts in the sea urchin egg; and though many have done so in the past, it is not necessary to conclude from the fact that p-amino benzoic acid antagonizes the sulfonamides, that the former acts in any way other than as do promoters in inorganic catalysis. The latter point is emphasized by the recent contribution by Eyster ('43). This investigator has shown that p-amino benzoic acid will antagonize the sulfonamide inhibition of methylene blue absorption by activated charcoal. Unless we are prepared to imagine that p-amino benzoic acid is normally concerned in some way with the absorption on charcoal, we cannot ascribe to p-amino benzoic acid in this case any more than the properties of a promoter. Perhaps in the last analysis some growth factors act merely as promoters.

There is then no reason for suspecting that the effect of sulfanilamide on bacteria is significantly different from its effect in the sea urchin egg. It is, consequently, very likely that in bacteria as in yeast, protozoa and the sea urchin egg, cell division will be found closely linked with a specific system of respiratory reactions and that, as in the sea urchin egg, inhibition of cell division by sulfonamides will be found to parallel inhibition of this activity system (cf 6).

SUMMARY

1 Sulfanilamide depresses the rate of cell division in the fertilized egg of the sea urchin, *Arbacia punctulata*, and simultaneously the rate of oxygen consumption is lowered. Complete suppression of division by this agent is associated with a 45% inhibition of oxygen consumption. Sulfanilamide does not inhibit oxygen consumption in the unfertilized egg.

2 Azide does not inhibit respiration in the unfertilized cells although it depresses both oxygen consumption and cell division in the fertilized egg. The maximum effect of azide in the latter case results in a lowering of the rate of oxygen consumption to approximately 50% of the normal value, and it is necessary to produce this maximum effect in order to stop cell division (data of Krahf et al).

3 When added to a preparation already maximally inhibited with azide, sulfanilamide cannot produce additional inhibition. Urethane, on the contrary, does depress further the respiration maximally inhibited by azide.

4 The effect of urethane on respiration in the presence of the maximum azide effect can be described by an expression of the mass law. The value of a in this equation is similar to that for the urethane inhibition of the respiration of the unfertilized egg, and to that deduced for the effect of urethane on the basal system of the fertilized egg.

5 These observations all suggest the conclusion that cell division depends upon the normal function of a discrete chain of respiratory reactions, the "activity" system. This system is normally responsible for approximately 50% of the total oxygen consumption of the fertilized egg but it is inactive in the resting cell. Inhibition of cell division by sulfanilamide as well as by narcotics and azide, accompanies inhibition of the activity system. It is undoubtedly the latter inhibition which brings about the depression of the rate of cell division. The significance of these findings with relation to the effects of the sulfonamides in bacteria is discussed.

Acknowledgement We wish to express our appreciation to the John and Mary R. Markle Foundation for the grant to one of us (K. C. F.) which, in part, made this research possible.

LITERATURE CITED

- (1) BALL, ERIC G. 1942 Oxidative mechanisms in animal tissues pp 16-33 in—A symposium on respiratory enzymes. University of Wisconsin Press, Madison.
- (2) EYSTER, H. C. 1943 Mechanism of sulfanilamide action and its interaction with p -amino benzoic acid. *J. Cell and Comp. Physiol.* 21, 191.
- (3) FISHER, KENNETH C. 1942 Narcosis. *Can. Med. Assoc. Journ.* 47, 414.

- (4) FISHER, KENNETH C., AND JOSEPH R. STERN. 1942 The separation of an activity metabolism from the total respiration of yeast by the effects of ethyl carbamate. *J. Cell. and Comp. Physiol.*, **19**, 109.
- (5) FISHER, KENNETH C., AND R. J. HENRY. 1944 The effects of urethane and chloral hydrate on oxygen consumption and cell division in the egg of the sea urchin. *J. Gen. Physiol.*, in press.
- (6) HENRY, R. J. 1943 *Bact. Revs.*, in press.
- (7) KORR, IRVIN MORRIS. 1937 Respiratory mechanisms in the unfertilized and fertilized sea urchin egg. A temperature analysis. *J. Cell. and Comp. Physiol.*, **10**, 461.
- (8) KRAHL, M. E., A. K. KELTCH, C. E. NEUBECK AND G. H. A. CLOWES. 1941 Studies on cell metabolism and cell division. V. Cytochrome oxidase activity in the eggs of *Arbacia punctulata*. *J. Gen. Physiol.*, **24**, 597.
- (9) ONNISBEE, RICHARD A., AND KENNETH C. FISHER. 1944 The effect of urethane on the consumption of oxygen and the rate of cell division in the ciliate, *Tetrahymena geleii*. *J. Gen. Physiol.*, in press.
- (10) THOMAS, J. A. 1941 *Compt. Rendu*, **213**, 890. (Physiol. Section, British Chemical Abstracts.)

INHIBITION OF CHOLINESTERASE ACTIVITY OF NERVOUS TISSUES BY ESERINE IN VIVO¹

G. L. CANTONI AND O. LOEWI

From Department of Pharmacology, New York University College of Medicine

Received for publication January 10 1944

Ever since the discovery of the inhibition of cholinesterase (ChE) activity by eserine, this alkaloid has been extensively used, both experimentally and therapeutically, in order to protect acetylcholine (Ach) from enzymatic destruction.

Provided that adequate concentrations of eserine are used it is possible to inhibit completely in isolated organs the cholinesterase activity. Extraction with solutions containing eserine has, therefore, often been used in order to determine the Ach content of tissues.

Ample evidence is available to indicate that also in vivo, the administration of eserine protects from enzymatic hydrolysis the Ach liberated from cholinergic fibers (1). However, so far as we know, it has not been shown whether it is possible, by adequate administration of eserine to inhibit completely the enzymatic destruction of Ach within tissues in vivo.

In the investigations here reported we have approached the question for the central nervous system (CNS) and the peripheral nerves.

For the CNS Gellhorn, Cortell and Feldman (2) have shown in rabbits that eserine may inhibit by 50% the ChE activity of the brain stem without grossly disturbing the nervous functions. They used, however, only relatively small doses of eserine and were not primarily concerned with obtaining a complete inhibition of ChE activity.

For the peripheral nerves it has not been shown whether eserine, administered by injection, enters the intact nerve fibers. Treatment of frog's isolated nerve with eserine results in practically no change in the action current (3). The question of whether it is possible to protect completely, by eserine administration, the Ach normally present in nerves seemed of particular interest with regard to the possibility, recently discussed by Nachmansohn and associates (4), that the release and hydrolysis of Ach may be the first events in a series of reactions responsible for the propagation of the impulse within the nerve itself.

METHODS Since both Ach and ChE are present in tissues Ach is completely or almost completely hydrolyzed when the tissues are ground in salt solutions and allowed to stand for a short while. On the other hand if eserine in sufficient concentration is added to the salt solution before the grinding no Ach is lost. We examined whether the same degree of protection of the Ach contained in nervous tissues could be attained by previous subcutaneous injection of eserine. We used frogs (*Rana pipiens* weighing from 55 to 80 Gm.) since they tolerate much larger doses of eserine than mammals.

The general procedure for the peripheral nerves was as follows: the two sciatic nerves were dissected clean from the point of emergence from the spinal column to the knee. rinsed

¹ Aided by a grant from the Rockefeller Foundation given to O. L.

once in bicarbonate free Ringer, dried rapidly with filter paper, placed in a tared vessel containing 0.4 cc. of bicarbonate free Ringer one with, one without Eserine, and weighed. The tissues and the solution were then transferred to a porcelain mortar where the tissues were finely cut up for 60 sec., ground with a minute amount of quartz sand for 60 sec., and incubated at 37° for 13 min. At the end of the incubation period, bicarbonate free Ringer containing eserine $1:10^{-4}$ was added to both nerves in order to stop the further enzymatic destruction of Ach. The tissue mash was transferred to centrifuge tubes and the volume adjusted with bicarbonate free Ringer containing eserine $1:10^{-4}$ to 3 cc. for each 100 mgm. of tissue. After centrifuging, the supernatant fluid was poured off and kept in the ice box until just before assaying, when it was diluted with Ringer containing bicarbonate.

Assays were made on the isolated frog's heart (Straub's preparation) and matched against Ach.-chloride solutions. A series of four curves was always taken in the order ABBA as recommended by Burn (5), to take into account incidental variations in the sensitivity of the preparation. The Ringer contained Na-Oleinate in a concentration $1:10^{-5}$ in order to keep the frog's heart beating optimally throughout the experiment (6). When eserine was injected, we used a 1% solution. This was injected into the abdominal lymph sac 30 min. before the dissection of the sciatics.

EXPERIMENTS. In the first series of experiments we used both of the sciatic nerves of single frogs to determine Ach. values; the treatment of the frogs and the sciatics was different for the three groups of this series. The details of the experimental procedure together with the results obtained are summarized in table 1. The experiments of Group I, confirming previous observations, indicate that by grinding in eserine free solution practically the whole of the Ach. contained in normal nerves is hydrolyzed by the ChE. In contrast to this, also confirming previous observations, the experiments of Group II indicate that the Ach. is preserved if the grinding is done in Ringer's containing eserine. The absolute values obtained for this group are of the same order of magnitude as those reported by Hellauer and Umrath (7) for the sciatics of European frogs. The animals of Group III had received eserine by subcutaneous injection. The dose of eserine was selected so as to result in a tissue concentration of approximately $1:10000$, assuming of course that the drug distributes uniformly throughout the tissues. The absorption of the injected fluid was found, in most cases, to have been complete in the thirty minutes allowed for it.

The frogs after the injection were apparently normal in their posture and behavior, the heart was beating normally and the muscles of the legs responded with contraction to the section of the nerve. In a few animals we tried to see whether we could detect any sign of impaired nerve conduction. By faradic stimulation of the peripheral end of the cut sciatic it was possible to elicit both simple muscle twitches and tetani. The average Ach. content of this group did not show any difference from that of Group II.

The experiments reported in table 1 were done using the two sciatics of single frogs for each experiment. Although both the single experiments and the average values for Groups II and III are well comparable, it seemed worth while to repeat the observations in more rigorously controlled experiments. In a new series this was done, determining the Ach. content of the two sciatics separately before and after the administration of eserine. The experimental procedure was as follows: Under light ether anaesthesia we removed from one side a section of

the sciatic from high in the thigh to the knee after tying the femoral artery to this side, the skin was then sutured. There was no hemorrhage. The sciatics were immediately ground in Ringer containing eserine. When the frog had recovered from the anaesthesia eserine was injected, and 30 min later the sciatics of the other side were removed under similar conditions, but extracted with eserine free Ringer. The results are given in table 2.

The Ach values of the nerves of the two sides are identical. Hereby the results obtained in the first series of experiments are confirmed. They indicate that after subcutaneous injections of adequate amounts of eserine the Ach of periph

TABLE 1
Acetylcholine content of frog's sciatic nerves

GROUP	NUMBER OF EXPERIMENTS	TREATMENT	ACH CONTENT
I	5	Control Ground and incubated in eserine free Ringer's	γ/gm 0.09 (0.005-0.22)
II	6	Control Ground and incubated in eserine Ringer's 1:10 ⁴	2.09 (1.2-4.0)
III	7	Sc injection of 7.5 mgm Eserine (1 mgm/10 Gm of body weight) ground and incubated in eserine free Ringer's	1.94 (1.15-3.0)

TABLE 2

	NO OF FROGS	ACH CONTENT γ/GM OF RIGHT SCIATIC NORMAL SIDE	ACH CONTENT γ/GM OF LEFT SCIATIC 30 AFTER INJECTION OF 7.5 MGm ESERINE
Exp 1	5	2.65	2.65
Exp 2	6	1.85	1.85

eral nerves is completely protected from enzymatic destruction by the ChE. It is well to mention here that Ach levels whether determined by the frog's heart, the leech's dorsal muscle, or the cat's blood pressure are accurate only to within 5-10%. In order to see whether eserine could reach the nerve fiber by diffusion, we performed a few experiments where we injected eserine in the usual dose and manner into frogs whose circulation had been stopped by means of a ligature placed around the ventricle. We found Ach values for the sciatic nerves of 0.01 gamma and 0.00 gamma/gm weight which indicates that diffusion of the injected eserine does not take place under these conditions.

EXPERIMENTS ON THE CNS. 30 min after the administration of eserine the brain stem was isolated and divided longitudinally in two halves that were incu

bated in Bicarbonate free Ringer, and Bicarbonate free Ringer with eserine 1:5000 respectively.

After incubation the water soluble Ach. was extracted with Ringer containing eserine in both cases. The eserine concentration had to be raised to 1:5000 (8). This method of extraction yields only between $\frac{1}{10}$ and $\frac{1}{5}$ of the total amount of Ach. contained in the CNS of the frog. (9). Loewi and associates (loc. cit.) however have shown that the water insoluble Ach. is resistant to the ChE. and therefore the Ringer extraction was chosen, as more suitable for our experiments. The results are summarized in table 3. After the injection of eserine, the average Ach. content of the half of the CNS that was incubated with eserine 1:5000 is 2.34 gamma/gm., weight whereas the corresponding halves incubated without eserine give a content of only 0.26 gamma/gm. weight or less. This indicates that in the case of the CNS we have been unable to inhibit the destruction of Ach. by means of subcutaneous injection of eserine.

TABLE 3
*Ach. content of frog's CNS following subcutaneous injection
of Eserine and incubation at 37° 15'*

NO. OF EXP.	TREATMENT	ACH. γ /GM.
6	$\frac{1}{2}$ CNS incubated with Ringer containing Es 1:5000	2.34 (1.25-3.00)
6	$\frac{1}{2}$ CNS incubated with Eserine free Ringer	0.26 (0.55-0.905)

DISCUSSION. Our failure to produce by eserine injection any protection of the Ach. of the CNS from enzymatic hydrolysis is probably due to the much higher degree of ChE. activity of the CNS as compared with the peripheral nerves. This explanation is supported by the observations of Hellauer (10) who has shown that in the frog the activity of the ChE. of the CNS is 4 to 5 times greater than that of the sciatic nerve per unit of weight.²

Our results on peripheral nerves on the other hand indicate that the ChE. of these tissues must have been inhibited to such a degree that within the limits discussed above the whole amount of Ach. present in nerves has been protected against the action of the enzyme. Yet there was no evidence of inhibition of the propagated impulse within the nerves.

How can these results be reconciled with Nachmansohn's suggestion according to which the propagated impulse is initiated by liberation and hydrolysis of Ach.?

In order to extract the Ach. it is of course necessary to grind up the nerve, thereby its structure is destroyed and eserine is allowed to diffuse to all elements of the nerve. One could, perhaps, assume that within the intact nerve the in-

² In a few experiments we tried to reach a higher eserine concentration by raising the dose to 15 mgm./Gm. body weight. Even with this very high dose we did not succeed in effectively protecting the Ach. from the ChE. of the CNS.

jected eserine does not reach the spots where the cholinesterase is located and active. We have no knowledge about these spots. From all the available literature (11) we do know, however, that eserine inhibits the cholinesterase activity in intact tissues. It can of course not be excluded that just nerve fibres present an exception from this general rule. As long as this question has not been settled, our results while they do not support, do not necessarily invalidate Nachmansohn's suggestion.

SUMMARY

1. The acetylcholine present in the central nervous system and in the peripheral nerves is completely or almost completely hydrolyzed during grinding and subsequent incubation of the tissues.

2. Subcutaneous injection of eserine even in large doses does not affect this process in the case of the central nervous system.

3. In contrast to this the acetylcholine of peripheral nerves is completely protected from hydrolysis by subcutaneous injection of eserine.

4. Propagation of impulses in such nerves is present.

REFERENCES

- (1) BROWN G. L. *Physiol. Rev.* 17: 485, 1937.
- (2) CORTELLI R., FELDMAN J. AND GELLHORN F. *Am. J. Physiol.* 132: 555, 1941.
- (3) COWAN S. L. *J. of Physiol.* 93: 215, 1938.
- (4) NACHMANSOHN D. AND MEYERHOF B. *J. Neurophysiol.* 4: 348, 1941.
NACHMANSOHN D., COX R. T., COATES C. W. AND MACHADO A. L. *J. Neurophysiol.* 5: 499, 1942.
- (5) BURNS J. H. *Biological Standardization*, Oxford Medical Publication, 1937.
- (6) LOEWI O. Unpublished experiments.
- (7) HELLAUER H. AND UMNATH K. *Zeitsche für Biol.*, 99: 624, 1939.
- (8) LOEWI O. AND HELLAUER H. *Pflügers Arch.* 244: 449, 1938.
- (9) LOEWI O. *Pflügers Arch.* 239: 430, 1937.
- (10) HELLAUER H. *Pflügers Arch.* 242: 382, 1939.
- (11) GLICK D. *Biol. Symp.* 5: 213, 1941.

ANESTHETIC ACTIVITY OF THE *CIS-TRANS* ISOMERS OF TRICHLOROETHYLIDENE GLYCEROL¹

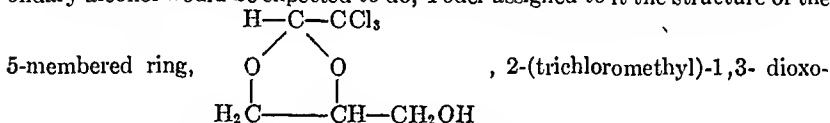
THOMAS C. BUTLER

*From the Department of Pharmacology, Vanderbilt University School of Medicine,
Nashville, Tennessee*

Received for publication January 22, 1944

Trichloroethylidene glycerol was first prepared by Yoder (1). He reported it to have "a marked and fleeting hypnotic action", but said nothing about the method of testing or the species of animal used. The preparation of the substance has since been described by Böcseken (2), by Hibbert, Morazain, and Paquet (3), and by Meldrum and Vad (4); but beyond Yoder's brief remark, I have failed to find any publication on its pharmacology.

Because his product reacted more readily with carbonyl chloride than a secondary alcohol would be expected to do, Yoder assigned to it the structure of the



There are four possible stereoisomers having the structure shown above (*cis* and *trans* forms, each a racemic pair). By mechanical separation of the crystals Hibbert *et al.* were able to isolate and characterize the two isomeric benzoates, but separation of the isomeric alcohols themselves has not hitherto been reported. By benzylation of trichloroethylidene glycerol, separation of the two crystalline benzoates, and hydrolysis of the benzoates, I have obtained sufficient quantities of the two isomeric forms of trichloroethylidene glycerol for pharmacological tests on mice. These two isomers are almost certainly the *cis-trans* isomers of 2-(trichloromethyl)-1,3-dioxolane-4-methanol and each is a racemic modification. In the absence of conclusive evidence as to which is the *cis* and which is the *trans* form, the lower melting alcohol will here be designated the α -form and the higher melting the β -form.

¹ This work was supported by a grant from the Mallinckrodt Chemical Works.

Methods *Preparation of trichloroethylidene glycerol* The method is essentially that used by Böeseken (2). To 92 Gm (1 mole) of glycerol was added 162 Gm (1.1 mole) of ebloral and 140 cc of concentrated sulfuric acid. The temperature rose to 100° and the mixture became homogeneous and turned brown. After $\frac{1}{2}$ hour, it was cooled, diluted with water, and extracted with chloroform. The chloroform extract was washed with water and sodium bicarbonate solution and dried with anhydrous sodium sulfate. The chloroform was then distilled off and the product distilled at a pressure of 7 mm. Yield 40 per cent.

Separation of the benzoates The benzylation was performed in the manner described by Hibbert *et al.* (3). The two crystalline benzoates were separated by a long series of crystallizations from hexane, benzene, and from methanol with seeding. Mechanical separation of large, well formed crystals was sometimes employed. The final melting points were 74.2–75.2° and 83.8–84.8°C (corrected). These melting points could not be changed by further crystallization from hexane or from methanol.

Hydrolysis of the benzoates The benzoate was dissolved in methanol and 2 equivalents of potassium hydroxide in methanol added. After 10 min. water was added to reduce the methanol concentration to 70 per cent. After this mixture had stood at room temperature for a day, most of the methanol was removed by distillation at reduced pressure and the remaining solution extracted with chloroform. The extract was washed with water, the chloroform evaporated off, and the trichloroethylidene glycerol distilled without ebullition in a microdistillation apparatus at a temperature of 100° and a pressure of less than 1 mm. The higher melting benzoate gave rise to the higher melting alcohol.

Although interconversion of *cis-trans* isomers would not be expected to take place readily in this type of compound, the following experiment was performed to rule out the possibility. Samples of 0.3 gm of each of the isomeric alcohols were rebenzoylated. In each case more than 75 per cent of the theoretical yield of benzoate was isolated in the form of crystals having the structure and melting point of the original form of benzoate from which the alcohol was derived. In neither case could any crystals of the other form be found. This indicates that the processes of hydrolysis and distillation did not produce any significant interconversion of the isomers.

Pharmacological tests Male white mice were used. All doses of all three drugs were given as freshly prepared solutions of 25 mgm per cc in distilled water. The intravenous injections, both in the anesthetic and lethal ranges, were given at a rate of 0.20 cc per min, the piston of the syringe being advanced at a constant rate by a machine. A mouse was considered anesthetized if it could not gain and maintain the standing posture when its tail was pinched vigorously. Median doses were estimated by interpolation on the assumption that the curve relating log dose to proportion affected is the integrated normal frequency curve.

Trichloroethylidene glycerol is a colorless, odorless, viscous liquid. Boiling point 130°/7 mm. $Sp\ gr\ \frac{20^\circ}{4^\circ} = 1.545$. Exposure to light causes decomposition with the liberation of hydrogen chloride. This decomposition is detectable after an exposure of only a few hours to bright sunlight, but a sample kept in the dark at room temperature for three months showed no evidence of decomposition. Some of the properties of the component isomers are shown in table 1. The β isomer is solid at ordinary room temperatures, but freezing is slow and uncertain unless the liquid is seeded. Although the melting points as shown in table 1 are far apart, the two isomers differ little if any in their water solubility, this being about 3 gm per 100 cc at 20° for each.

The anesthetic and lethal effects of the two isomers of trichloroethylidene glycerol following intravenous and intraperitoneal injection in mice have been

studied. Studies of the corresponding effects of tribromoethanol have been made at the same time for comparison, this drug being also a halogenated alcohol and resembling the trichloroethylidene glycerols in activity and duration of action perhaps as closely as any other familiar drug.

The results are to be found in tables 2, 3, and 4. There is little difference in the effects produced by the two isomers. The estimated median anesthetic and lethal doses by both routes of administration are somewhat higher for the β -isomer, but only with the intraperitoneal anesthetic doses is the difference statistically significant. By both routes the trichloroethylidene glycerols are little less active as anesthetics than tribromoethanol, but their lethal doses are notably higher than the corresponding values for tribromoethanol.

TABLE 1
Properties of the two isomeric forms of trichloroethylidene glycerol

ISOMER	CHLORINE*	n_D^{20}	MELTING RANGE
	<i>per cent</i>		$^{\circ}\text{C.}$
α	47.1	1.5038	-50 to -35
β	47.3	1.5035	+19 to +28

* Theoretical ($\text{C}_3\text{H}_7\text{O}_3\text{Cl}_3$): 48.0 per cent.

TABLE 2
Anesthetic and lethal effects produced in mice by the intravenous injection of the isomeric trichloroethylidene glycerols (α - and β -forms) and of tribromoethanol (TBE)

DOSE	NUMBER OF MICE ANESTHETIZED/TOTAL			DOSE	NUMBER OF MICE KILLED/TOTAL		
	α	β	TBE		α	β	TBE
<i>mgm. per kgm.</i>				<i>mgm. per kgm.</i>			
91	1/15	4/15	5/15	265			6/15
100	8/15	5/15	7/15	304			10/15
110	9/15	7/15	9/15	463	4/15	5/15	
121	11/15	10/15		532	10/15	8/15	

Both isomers produce a quiet anesthesia with good relaxation in mice. The onset of anesthesia is immediate after an intravenous injection. The duration of anesthesia after small intravenous doses is quite short, but mice given intraperitoneal doses near the lethal range may be anesthetized for several hours. The median recovery time in a series of 21 mice receiving 200 mgm. per kgm. of the α -isomer intravenously was 50 sec. In a series of the same number receiving the same dose of the β -isomer, it was 20 sec. There were some deaths in the group of 21 that were given 200 mgm. per kgm. of tribromoethanol, but the eleventh mouse to recover did so in 140 sec.

The pharmacological study of geometrical isomers has not had the same obvious theoretical appeal as has that of optical isomers. Few investigations have been directed specifically to this end, and I know of no attempt at a com-

plete collection of the scattered observations that are in the literature. So far as I know the only other *cis trans* isomers that have been compared as general anesthetics are the 1,2 dichloroethylenes. The *cis* compound is reported to be considerably more active as a narcotic than the *trans*, and the side actions are different (5). Among central nervous system depressants of another type, morphine and its derivatives, geometrical isomerism has been studied. In those compounds change of the geometrical configuration of the alcoholic hydroxyl group is known to lead to qualitative and quantitative changes in action (6). Several geometrically isomeric local anesthetics have been tested. Little differ-

TABLE 3

Anesthetic and lethal effects produced in mice by the intraperitoneal injection of the isomeric trichloroethylidene glycerols (α and β forms) and of tribromoethanol (TBE)

DOSE	NUMBER OF MICE ANESTHETIZED/TOTAL			DOSE	NUMBER OF MICE KILLED/TOTAL		
	α	β	TBE		α	β	TBE
mgm per kgm				mgm per kgm			
200			4/15	532			7/20
230	4/15		0/15	612			19/20
255	0/15	3/15	11/15	809	3/15	1/15	
304		9/15		931	8/15	4/15	
				1070		14/15	

TABLE 4

Median anesthetic doses (AD 50) and median lethal doses (LD 50) with their standard errors, calculated from the data of tables 2 and 3

DRUG	INTRAVENOUS			INTRAPERITONEAL		
	AD 50	LD 50	LD 50 AD 50	AD 50	LD 50	LD 50 AD 50
α	105.2 \pm 3.2	502 \pm 16	4.78 \pm 0.24	255 \pm 9	920 \pm 32	3.61 \pm 0.18
β	110.4 \pm 4.6	518 \pm 31	4.69 \pm 0.35	295 \pm 9	950 \pm 33	3.26 \pm 0.15
TBE	102.1 \pm 5.0	279 \pm 14	2.73 \pm 0.19	235 \pm 10	546 \pm 9	2.32 \pm 0.11

ence in activity was found between the optically isomeric β -eucaines and their geometrical isomers, the *iso* β eucaines (7). Some difference was found between tropacocaine (benzoylpseudotropine) and its isomer, benzoyltropine (8). The optically isomeric cocaines are also reported to differ somewhat from their geometrical isomers, the pseudococaines (8). Probably the most striking pharmacological differences between *cis trans* isomers are to be found in the parasympatholytics and in the sex hormones. The geometrical isomers of atropine and homatropine are not mydriatic (9). There is a similar difference in mydriatic action between the mandelates of the *cis trans* forms of the N-methylnylidinetonalkylamines (10). In the steroid estrogens and androgens the activity is dependent to a great extent on the spatial configuration of the C_{17} -OH with

respect to the $C_{13}-CH_3$. Compounds in which the $-OH$ occupies a *trans* position are much more potent than the corresponding *cis* compounds (11, 12).

Among the substances producing pure, "typical" narcotic effects, there is little evidence of structural specificity. Similar narcotic effects are produced by a wide variety of chemical classes, and narcotic activity is more closely correlated with physical properties than with chemical structure. Enantiomorphic narcotics may have identical activity (13). Although the physical properties of *cis-trans* isomers are not identical, they may differ but little (as the water solubility of this pair). The mechanism of the narcosis produced by these compounds is probably dependent upon physical properties which, like water solubility, do not differ greatly rather than upon the spacial configuration of the molecule.

Even though trichloroethylidene glycerol is a mixture of isomers the proportions of which are unknown and perhaps even unreproducible, the close similarity in pharmacological action of the components indicates that this would probably be of no importance in any practical use of the unseparated mixture.

SUMMARY

Trichloroethylidene glycerol has been separated into the two component *cis-trans* isomers (each a racemic modification). These have been tested on mice in comparison with tribromoethanol. Both isomers produce a quiet anesthesia of brief duration, and they are nearly equal in activity. They are little less active as anesthetics both by the intravenous and by the intraperitoneal route than is tribromoethanol, but their lethal doses by both routes are conspicuously higher than the corresponding values for tribromoethanol.

REFERENCES

- (1) YODER, L., *J. Am. Chem. Soc.*, **45**: 475, 1923.
- (2) BÖESEKEN, J., *Verslag Akad. Wetenschappen Amsterdam*, **35**: 1084, 1926.
- (3) HIBBERT, H., MORAZAIN, J. G., AND PAQUET, A., *Canad. J. Research (Section B)*, **2**: 131, 1930.
- (4) MELDRUM, A. N., AND VAD, G. M., *J. Indian Chem. Soc.*, **13**: 118, 1936.
- (5) LEHMANN, K. B., AND SCHMIDT-KEHL, L., *Arch. f. Hyg.*, **116**: 131, 1936.
- (6) SMALL, L. F., EDDY, N. B., MOSETTIG, E., AND HIMMELSBACH, C. K., *Studies on Drug Addiction* (Suppl. No. 138 to the Public Health Reports, U. S. Govt. Printing Office, 1938).
- (7) KING, H., *J. Chem. Soc.*, **125**: 41, 1924.
- (8) GOTTLIEB, R., *Arch. f. exper. Path. u. Pharmacol.*, **97**: 113, 1923.
- (9) LIEBERMANN, C., AND LIMPACH, L., *Ber. deut. chem. Ges.*, **25**: 927, 1892.
- (10) HARRIES, C., *Ber. deut. chem. Ges.*, **29**: 2730, 1896.
- (11) WHITMAN, B., WINTERSTEINER, O., AND SCHWENK, E., *J. Biol. Chem.*, **118**: 789, 1937.
- (12) RUZICKA, L., AND KÁGI, H., *Helv. Chim. Acta*, **20**: 1557, 1937.
- (13) BUTLER, T. C., AND DICKISON, H. L., *THIS JOURNAL*, **69**: 225, 1940.

EFFECT OF TYROSINASE ON PHENETHYLAMINE DERIVATIVES¹

LOWELL O. RANDALL AND GEORGE H. HITCHINGS

The Wellcome Research Laboratories, Tuckahoe, N. Y.

Received for publication August 10, 1943

The present study deals with the oxidation of a series of phenethylamine derivatives as catalyzed by tyrosinase, and the possible correlation of the rates of oxidation with the chemical structure and the physiological activity of the amines. In addition to the primary amines, the series includes the secondary, tertiary and quaternary derivatives of phenethylamine, the 2, 3 and 4 mono-hydroxyphenethylamines and the 2,3 and 3,4-dihydroxyphenethylamines and epinephrine. The pharmacological activities of the secondary amines were described by Hjort (1). Most of the compounds were synthesized in these laboratories by J. S. Buck and associates.

EXPERIMENTAL

METHODS Stable tyrosinase preparations were made from extracts of the cultivated white edible mushroom by fractional precipitation with ammonium sulfate (2). Two such preparations were studied in detail. Their activities were assayed using catechol and cresol as substrates by the method of Alles *et al.* (3). The rates of oxidation of the amines were determined in the Warburg apparatus using 1 ml. of enzyme solution, 0.5 ml. of 0.05 molar substrate and 1 ml. of phosphate gelatin buffer at pH 7.0 at a temperature of 30°C. with shaking at the rate of 120 oscillations per minute. The oxygen consumption was measured at convenient intervals until at least 50 microliters of oxygen had been consumed. Determinations of the rates of oxygen uptake in the absence of enzyme showed that the 3,4-dihydroxy derivatives consumed about 0.1 microliter and the 2,3-dihydroxy derivatives about 0.3 microliter per minute. The blank value with each of the other amines was negligible. When it was significantly large, the value for the autooxidation was subtracted from the total oxygen uptake in the calculation of the rate of enzymatic reaction.

RESULTS Figure 1 summarizes graphically the initial rates of oxidation of the various substrates using enzyme T₁ as the catalyst. For each point the ordinate gives the oxygen consumption in microliters and the abscissa the time in minutes. In each instance the oxygen consumption due to tyrosinase appeared to be a linear function of time during initial stages of the reaction (consumption of 50 microliters or 0.179 atoms of oxygen per molecule of amine). The complex nature of the reactions involved prevented a simple formulation of the reaction rates when oxygen consumption was followed to higher levels.

In general, the primary and secondary amines of any series were oxidized at approximately equal rates. Likewise, the tertiary and quaternary derivatives were oxidized at similar rates but more slowly than the primary and secondary amines. For example, the primary and secondary 3,4-dihydroxyphenethyl amines took up 8.2 μ l./min. while the tertiary and quaternary derivatives con-

¹ Presented in part before the Division of Biological Chemistry of the American Chemical Society at Detroit, April 12-16, 1943.

sumed $7.0 \mu\text{l/min}$. The 4-hydroxyphenethylamine and its secondary derivative were oxidized at the rate of $3.0 \mu\text{l/min}$, while the tertiary and quaternary derivatives had the slower rate of $1.7 \mu\text{l/min}$. The primary and secondary 2,3-dihydroxy derivatives consumed $0.8 \mu\text{l/min}$, and the tertiary and quaternary derivatives were oxidized at half this rate.

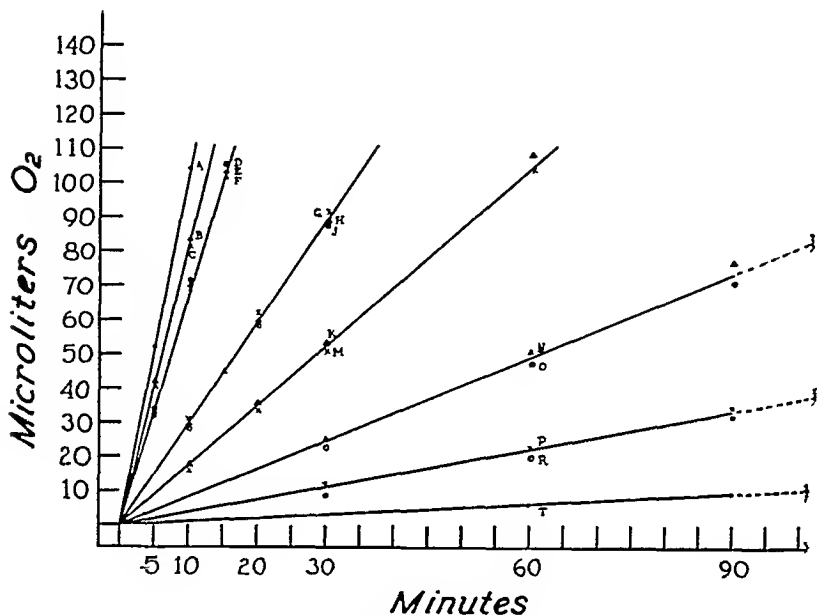


FIG 1 TYROSINASE AND PHENETHYLAMINE DERIVATIVES

Oxygen uptake in microliters against time in minutes of equimolar concentration of amines as catalyzed by tyrosinase. The substrates included A, catechol, B, 3,4 dihydroxy phenethylamine hydrochloride, C, 3,4 dihydroxy phenethylmethylamine hydrochloride, D, 3,4 dihydroxy phenethyltrimethylamine hydrochloride, E, 3,4 dihydroxy phenethyltrimethyl ammonium chloride, F, cresol, G, 4 hydroxyphenethylamine hydrochloride, H, 1 epinephrine hydrochloride, J, 4 hydroxyphenethylmethylamine hydrochloride, K, 4 hydroxyphenethyltrimethylamine hydrochloride, L, 4 hydroxyphenethyltrimethyl ammonium chloride, M, 4 hydroxyphenethylmethylamine hydrochloride, N, 2,3 dihydroxyphenethylamine hydrochloride, O, 2,3 dihydroxyphenethylmethylamine hydrochloride, P, 2,3 dihydroxyphenethyltrimethylamine hydrochloride, Q, 2,3 dihydroxyphenethyltrimethyl ammonium chloride, R, 3 hydroxyphenethylamine hydrochloride, T, 3 hydroxyphenethylamine hydrochloride.

Among the diphenolic amines, the 3,4-dihydroxy derivatives were oxidized more rapidly than the 2,3-dihydroxy compounds. Epinephrine was oxidized at less than half the rate of epinine.

Among the monophenolic amines, the 4-hydroxyphenethylamines were oxidized more rapidly than the 3-hydroxyphenethylamine while the 2-hydroxy- and the unsubstituted phenethylamines were not oxidized at all. Alles *et al* (3) concluded that the 3-hydroxyphenethylamines are not oxidized by tyrosinase. However, in the present experiment the primary amine of this series was oxidized.

at a significant rate though a period of about 8 hours was required for the consumption of 50 μ l of oxygen. No oxidation was observed with the secondary, tertiary and quaternary derivatives.

TABLE I
Rate of oxidation of phenethylamine derivatives by tyrosinase*

NO	NAME OF COMPOUND	T ₁	T ₂	RATIO— T / T ₁	COLOR OF SOLUTION	COLOR OF PRECIPITATE
		Rate of Oxygen Consumpt on (μ l /m n)				
	Catechol	10.4	16.0	1.5	Red	None
	Cresol	6.8	1.1	0.16	Red	None
21	1 Epinephrine hydrochloride	3.0	1.6	0.53	Red orange	Black
69	3,4 Dihydroxyphenethylamine hydrochloride	8.3	13.1	1.6	Red brown	Black
22	3,4 Dihydroxyphenethylmethylaniline hydrochloride	8.1	12.8	1.6	Red brown	Black
68	3,4 Dihydroxyphenethyldimethylaniline hydrochloride	7.1	8.0	1.2	None	None
818	3,4 Dihydroxyphenethyltrimethyl ammonium chloride	7.0	8.7	1.2	Red orange	None
696	2,3 Dihydroxyphenethylamine hydrochloride	0.9	0.4	0.44	Red brown	Brown
38	2,3 Dihydroxyphenethylmethylaniline hydrochloride	0.8	0.4	0.50	Red brown	Brown
688	2,3 Dihydroxyphenethyldimethylaniline hydrochloride	0.4	0.2	0.50	Red brown	None
817	2,3 Dihydroxyphenethyltrimethyl ammonium chloride	0.3	0.2	0.67	Red brown	None
695	4 Hydroxyphenethylamine hydrochloride	3.1	2.2	0.71	Red brown	Black
32	4 Hydroxyphenethylmethylaniline hydrochloride	2.9	1.8	0.62	Red brown	Black
687	4 Hydroxyphenethyldimethylaniline hydrochloride	1.7	0.8	0.49	None	None
816	4 Hydroxyphenethyltrimethyl ammonium chloride	1.8	0.8	0.47	Brown	None
694	3 Hydroxyphenethylamine hydrochloride	0.1			Red brown	Black

* No oxidation was observed with the following compounds: 3 hydroxyphenethylmethylaniline hydrochloride, 3 hydroxyphenethyldimethylaniline hydrochloride, 3 hydroxyphenethyltrimethyl ammonium chloride, 2 hydroxyphenethylamine hydrochloride, 2 hydroxyphenethylmethylaniline hydrochloride, 2 hydroxyphenethyldimethylaniline hydrochloride, 2 hydroxyphenethyltrimethyl ammonium chloride, phenethylamine hydrochloride, phenethylmethylaniline hydrochloride, phenethyldimethylaniline hydrochloride and phenethyltrimethyl ammonium chloride.

Table I gives data which allow a comparison of the effects of two enzyme preparations which differ widely in the ratio of catecholase to cresolase activity. Enzyme T₁ contained 1.04 units of catecholase and 0.68 units of cresolase activity.

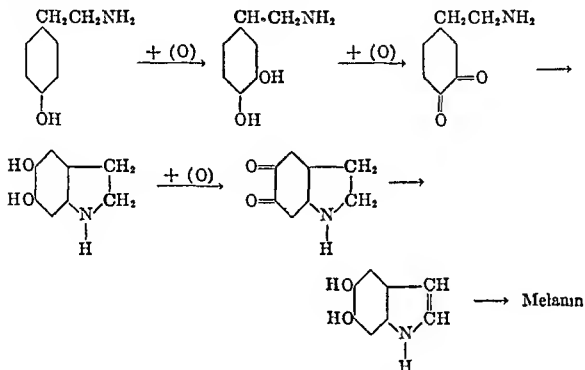
per ml., while for T_2 the corresponding values were 1.6 and 0.11 respectively. Thus the enzyme T_2 had a catecholase activity 1.5 times that of T_1 while the cresolase activity was only one-sixth. It was observed that only the 3,4-dihydroxyphenethylamines were oxidized more rapidly in the presence of T_2 than T_1 . Epinephrine, the 2,3-dihydroxy and the 4-hydroxy derivatives were oxidized less rapidly with T_2 as catalyst. This suggested that the rates of oxidation of the latter compounds were closely dependent on the cresolase activity of the preparation in contrast to the former which were affected primarily by the catecholase activity. Using either T_1 or T_2 epinephrine was oxidized at a rate less than half that of epinine. This result differs from the findings of Alles (3) who found the two substrates to be oxidized at nearly equal rates. Nearly equal rates of oxidation of epinine and epinephrine were found when mushroom press juice purified by a single ammonium sulfate precipitation was used as a source of enzyme. However, when such enzyme preparations were diluted with water the rate of oxidation of epinephrine diminished more rapidly than that of epinine. It appears, therefore, that extraneous substances in the enzyme preparation can affect the rate of oxidation of one or both these substances.

The colors of the oxidation products (Table 1) indicate that a variety of courses of oxidation occur. Thus the primary and secondary 3,4-dihydroxy and the 4-hydroxy derivatives yield reddish solutions and finally black precipitates. The tertiary amines do not form colored products, while the quaternary salts yield reddish solutions. The primary and secondary 2,3-dihydroxy derivatives give brown precipitates but the tertiary and quaternary derivatives give no precipitates.

Table 2 contains the results of studies on the course of oxidation of the phenethylamines by tyrosinase over a period of 48 hours. The enzyme used for the study was the same preparation as the T_2 of previous experiments but 10 times as concentrated. The substrate concentration was 0.5 ml. of 0.005 molar amine in a volume of 2.5 ml., i.e. one-tenth the concentration used earlier. The other conditions of the experiment were the same as described above.

The initial rates of oxidation in these experiments were not markedly different from those reported in Table 1, since the decreased concentration of substrate was balanced by an increased concentration of enzyme. Moreover, the relative rates of oxidation of the various substrates were similar. However, as the oxidation continued changes in the relative rates of oxidation were observed. Thus epinephrine and the primary and secondary 4-hydroxy phenethylamines which were oxidized initially at rates lower than those of the 3,4-dihydroxyphenethylamines, later were oxidized more rapidly and in 48 hours had consumed more oxygen. The 4-hydroxy derivatives usually consumed more oxygen than the corresponding 3,4-dihydroxy derivatives, while the latter consumed more than the 2,3-dihydroxy derivatives. The primary, secondary, tertiary and quaternary derivatives of the 2,3-dihydroxy compounds consumed nearly equivalent amounts, whereas among the 4-hydroxy and 3,4-dihydroxy compounds, the primary and secondary derivatives consumed more than the tertiary amines and the latter more than the quaternary salts. It is thus apparent that no simple explanation will fit all the types of oxidation.

DISCUSSION The mechanism of oxidation of tyramine by tyrosinase was worked out by Raper and associates (4). The principal steps in the oxidation are shown below



Tyramine is first oxidized to 3,4-dihydroxyphenethylamine, which is further oxidized to the corresponding orthoquinone. Then the ring closure to the 5,6-dihydroxy-dihydroindole occurs. The latter is further oxidized to the corresponding orthoquinone. A second internal oxidation reduction results in the indole derivative which gives rise to melanin.

A similar series of chemical changes has been adopted by various workers to explain the route of oxidation of similar primary and secondary amines. The oxidation of tertiary and quaternary derivatives reported here cannot follow this path of oxidation because such derivatives cannot cyclize like the primary and secondary amines. Therefore, unless an improbable demethylation occurs, the formation of indole derivatives is precluded. The failure of this step to occur is confirmed, in part, by the absence of melanin from the oxidation products (Table 1). Nevertheless the tertiary and quaternary amines consume from 3.1 to 5.6 atoms of oxygen in a 48 hour period. Therefore there must be open alternative routes of oxidation such as further oxidation of the benzene ring.

The behavior of epinephrine is somewhat anomalous. In two respects, at least, it resembles the 4-hydroxyphenethylamines more closely than the 3,4-dihydroxyphenethylamines, *i.e.* the initial rate of oxidation is relatively more greatly affected by the cresolase than by the catecholase activity of the enzyme preparation and the course of oxidation over an extended period is like that of the 4-hydroxy compounds.

There appears to be no correlation between the rate of tyrosinase oxidation and the physiological activity of the amines. The 3,4-dihydroxy compounds are more readily oxidized by tyrosinase than the 2,3-dihydroxy derivatives and

possess greater activities as pressors. On the other hand, epinephrine is oxidized at only half the rate of epinine but has 10 times the pressor activity (1). Moreover, 3-hydroxyphenethylmethylamine is oxidized at less than $\frac{1}{10}$ the rate of the 4-hydroxy compound but is twice as potent as a pressor agent (1). The susceptibility to attack by tyrosinase and the ability to act as pressor agents, therefore, appear to depend primarily on different molecular configurations.

TABLE II
Total oxidation of phenethylamine derivatives by tyrosinase

NO.	NAME OF COMPOUND	MICROLITERS OF OXYGEN									ATOMS O PER MOLECULE
		5 min	10 min.	30 min.	60 min.	2 hr.	4 hr.	8 hr.	24 hr.	48 hr.	
21	l-Epinephrine hydrochloride	13	27	56	70	114	129	143	169	188	6.7
697	3,4-Dihydroxyphenethylamine hydrochloride	48	72	91	97	104	109	116	129	153	5.5
22	3,4-Dihydroxyphenethylmethylamine hydrochloride	51	72	94	98	101	104	112	133	157	5.6
689	3,4-Dihydroxyphenethyldimethylamine hydrochloride	26	28	31	36	44	49	64	116	134	4.8
818	3,4-Dihydroxyphenethyltrimethyl ammonium chloride	28	35	42	46	52	54	58	81	97	3.5
696	2,3-Dihydroxyphenethylamine hydrochloride		8	21	24	31	37	47	65	88	3.1
38	2,3-Dihydroxyphenethylmethylamine hydrochloride		7	23	37	50	62	70	88	107	3.8
688	2,3-Dihydroxyphenethyldimethylamine hydrochloride		5	14	23	35	39	42	68	91	3.2
817	2,3-Dihydroxyphenethyltrimethyl ammonium chloride		4	12	20	34	38	41	75	87	3.1
695	4-Hydroxyphenethylamine hydrochloride	18	42	101	126	131	135	140	158	171	6.1
32	4-Hydroxyphenethylmethylamine hydrochloride	15	39	95	115	119	123	129	148	163	5.8
687	4-Hydroxyphenethyldimethylamine hydrochloride		6	11	25	45	59	82	131	157	5.6
816	4-Hydroxyphenethyltrimethyl ammonium chloride		4	10	20	30	35	45	66	87	3.1

SUMMARY

The initial rate of oxidation of a series of phenethylamine derivatives as catalyzed by tyrosinase has been studied.

The primary and secondary derivatives of any series were oxidized more rapidly than the tertiary and quaternary derivatives.

The 3,4-dihydroxyphenethylamines were oxidized more rapidly than the 2,3-dihydroxy derivatives or epinephrine.

The 4-hydroxyphenethylamines were oxidized more rapidly than the 3-hy-

droxyphenethylamine. The 2-hydroxy and unsubstituted phenethylamines, and the N-substituted 3-hydroxy derivatives were not oxidized at all.

There was no correlation of ease of oxidation with the physiological activity of the amines.

REFERENCES

- (1) HJORT, A. M., *THIS JOURNAL*, 52: 101, 1931.
- (2) TENENBAUM, L. E., AND H. JENSEN, *J. Biol. Chem.*, 145: 293, 1942.
- (3) ALLES, G. A., C. L. BLOHM AND P. R. SAUNDERS, *J. Biol. Chem.*, 144: 757, 1942.
- (4) DULIÈRE, W. L., AND H. S. RAPER: *Biochem. J.*, 24: 239, 1930.

AMIDES, AMINES AND RELATED COMPOUNDS AS DIURETICS

WERNER L. LIPSCHITZ AND ZAREH HADIDIAN

From The Lederle Laboratories, Pearl River, N. Y.

Received for publication December 2, 1943

In a previous paper (1) a method using rats was described for the bioassay of diuretics. With this method it is possible not only to estimate the diuretic activity of drugs from their dose-action curves but also to obtain some information on side-actions produced by them. With urea as the reference standard, the activities of several known diuretics (salts, xanthine derivatives, metal organic compounds) were determined. The sequence of activities among these compounds as determined by the rat method was found to be the same as that calculated from the average human therapeutic doses.

The fact that urea, (activity 1), biuret (1.34), and the xanthine derivatives (7.2 to 115), contain the same group $\text{=N}-\text{C}-\text{N=}$ at least once in the mole-

cule and are diuretically active encouraged a systematic search among amides and amines for substances with high diuretic activity. Seventy substances were thus studied in order to determine 1) their diuretic potency with reference to urea, and 2) side-actions. The procedure used in almost every instance was the same as that used in the study of the known diuretics, and is described in detail in the previous paper.

The results obtained are presented in tables 1 and 2. In table 1 are given the activity, the diuretic dose range utilized for the computation of the activity, the mean log action (\bar{y}) at mean log dose (\bar{x}) of the substance and of the standard, the total dose-range tested, and the side-actions. In table 2 are presented more detailed data on several substances found to possess high diuretic activities, with the statistical analysis of the data.

The results given in table 1 show that, in principle, acid amides are diuretically active but that simple amides are weaker diuretics than urea, and with increasing molecular weight the potencies of the homologues of acetamide decrease. The introduction of an OH-group in the α -position enhances the diuretic activity of the acid amides; and again the higher homologues are less potent. The furoyl group in the α -position enhances diuretic activity more than the hydroxy-group. Acetamidine is more potent than the corresponding amide or hydroxy-amide. Urea and simple urea-derivatives (thio-, methylated, urethane) are about equally active but doubling the urea chain yields substances of somewhat greater activity. Among the cyclic compounds containing the $\text{=N}-\text{C}-\text{N=}$

group at least once, there were found three substances which equal or even surpass the xanthine diuretics in activity: melamine, adenine and formoguanamine. Many closely related compounds, on the other hand, show no or only unimportant diuretic activity.

TABLE I

*Diuretic activity and side actions of the substances tested**

NO	SUBSTANCE	USEFUL DIURETIC DOSE RANGE (TOTAL DOSE RANGE TESTED)	NO OF EXPTS UTILIZED FOR COMPUTATION	MEAN LOG ACTION \bar{y} MEAN LOG DOSE \bar{x}		ACTIVITY OF SUB- STANCE	OBSERVATIONS ABOUT MODE OF ACTION AT mM/kg
				Sub stance	Urea		
	<i>Aliphatic amides</i>	mM/kg					
1	Formamide	27.8 - 111.2	4			0.2	
2	Acetamide	21.15 - 42.3	2	310 1 476	375 1 248	0.15	
3	N-Diethylacet- amide	(4.35 - 17.39)	2			0	anesthetic, 17.4 mM lethal in 25%
4	Propionamide	13.68 - 34.2 (13.68 - 51.3)	2	135 1 335	269 1 145	0.4	51.3mM narcotic
5	n-Butyramide	(14.35 - 28.7)	1			0	anesthetic
6	i-Butyramide	(28.73 - 43.1)	1			0	anesthetic
7	Malonamide	10.42 - 34.72	5	241 1 250	200 1 132	1.12	
8	Succinamide	(5.38 - 21.53)	2			0	
9	Ethylsulfonamide	11.47 - 22.93	2	525 1 210	520 1 248	1.14	
	<i>Hydroxy amides</i>						
10	Glycylamide	8.32 - 16.64	3	375 1 071	417 1 310	1.5	
11	Lactamide	7.02 - 14.05 (7.02 - 28.1)	2	290 1 098	340 1 248	1.57	
12	1-Malicethylester- amide	4.60 - 7.76	1	567 779	588 1 208	2.51	
13	1-Malamide	0.46 - 26.39	2	083 1 235	280 1 271	0.7	
14	d-Tartramide	8.44 - 16.88	2	204 1 075	320 1 097	0.5	
15	meso-Tartramide	8.45 - 16.89 (8.45 - 25.34)	2	355 1 122	435 1 213	0.7	
16	Gluconamide	(6.41 - 12.82)	1			0	
	<i>Cyclic amides</i>						
17	α -Furoamide	0.70 - 2.81 (0.70 - 11.25)	3	401 197	317 1 208	12.8	5.63 mM toxic, depressant
18	Tetrahydrofuro- amide	2.17 - 21.74	3			1.235	diuretic action prolonged over 5 hours
	<i>Urea derivatives</i>						
19	Urea	8.3 - 50.0	many			1.0	>35 mM 150- 195% of fluid fed excreted
20	Semicarbazid HCl	0.336 - 1.12 (0.336 - 22.4)	2			0	2.24 mM convul- sions

TABLE 1—Continued

NO.	SUBSTANCE	USEFUL DIURETIC DOSE RANGE (TOTAL DOSE RANGE TESTED)	NO. OF EXPTS. UTILIZED FOR COMPUTATION	MEAN LOG ACTION \bar{y} MEAN LOG DOSE \bar{x}		ACTIVITY OF SUB- STANCE	OBSERVATIONS ABOUT MODE OF ACTION AT mM./kg.
				Sub- stance	Urea		
		mM./kg.					
21	Biuret	6.0 - 12.02	4	.186 .930	.292 1.248	1.34	
22	Aminobiuret HCl	0.81 - 2.02 (0.81 - 4.05)	3	.154 .108	.241 1.208	9.1	2.02 mM: convul- sions, labored res- piration; 4.05 mM: lethal in 50%
23	Carbonyl-diurea	(8.56 - 25.69)	2			0	
24	Thiourea	13.14 - 26.28 (13.14 - 32.88)	4	.462 1.270	.429 1.208	1.1	
25	N-monomethyl- urea	16.9 - 33.8	3	.315 1.377	.214 1.222	0.8	
26	as-Dimethylurea	8.51 - 21.42	3	.317 1.141	.294 1.235	1.7	
27	sym-Dimethyl- urea	14.19 - 28.38	2	.492 1.271	.538 1.341	1.0	
28	Tetramethylurea	1.61 - 5.38 (1.61 - 21.51)	3	.231 .503	.372 1.188	4.7	>5.4 mM: anaes- thetic
29	Acetylurea	(24.51)	2			0	crystalluria
30	Hydantoin	7.5 - 25.0	3	.575 1.073	.431 1.273	2.5	
31	Guanylurea sul- fate	(3.7 - 7.4)	2			0	(antidiuretic?)
32	Allylurea	(25.0)	1			0	narcotic, toxic
33	Urethane	8.43 - 12.64	2	.167 1.026	.286 1.097	1.0	anaesthetic
34	N-methylurethane	(6.07 - 24.27)	1			0	24.3 mM: anaes- thetic
<i>Amidines:</i>							
35	Acetamidine HCl	1.32 - 2.64 (1.32 - 26.4)	8	.2663 .272	.348 1.216	6.5	26.4 mM: labored respiration, con- vulsions, death; lungs hemor- rhagic
36	Creatinine	8.84 - 22.1	2	.291 1.146	.375 1.164	0.7	
37	Methylguanidine sulf.	0.51 - 2.05 (0.51 - 8.18)	4	.111 .054	.237 1.188	13.4	1.54 mM: toxic; 2.05 mM: lethal
38	Diguanide sulf.	4.4 - 6.28 (4.4 - 12.56)	2	.288 .721	.360 1.120	1.95	8.8 mM: lethal
<i>Amines:</i>							
39	Ethylendiamine (HCl) ₂	0.47 - 2.35 (0.47 - 18.8)	4	.240 .073	.320 1.178	8.7	If fluid was not neutralized: diar- rhea
40	Ethanolamine (HCl)	5.15 - 12.81 (5.15 - 25.63)	4	.442 .819	.389 1.276	3.2	

TABLE 1—Continued

NO	SUBSTANCE	USEFUL DIURETIC DOSE RANGE (TOTAL DOSE RANGE TESTED)	NO. OF EXPTS UTILIZED IN COMPUTATION	MEAN LOG ACTION \bar{y}		ACTIVITY OF SUB- STANCE	OBSERVATIONS ABOUT MODE OF ACTION AT mM/kg
				Sub- stance	Urea		
		mM/kg					
41	N-acetyl ethanol- amine	9.71 - 19.42	2	665 1.118	597 1.313	1.9	
42	l-Asparagine	(9.5 - 19.0)	1			0	
43	Glycinanhydride	(11.0 - 22.0)	1			0	crystalluria
	<i>Pyrimidine derivatives</i>						
44	Guanine HCl	(3.04 - 6.08)	1			0	
45	Uracil	6.70 - 22.32	4	397 1.103	375 1.203	1.7	6.7 mM crystal- luria, 17.9 mM bloody urine
46	5 Amino uracil	4.92 - 19.67	2	113 993	423 1.208	0.2	—, urine brown
47	Uramil	(4.37 - 17.47)	2			0	—, urine red
48	Isocytosine	2.14 - 21.36	6	304 900	294 1.098	2.6	crystalluria
49	Thiamin HCl (Vitamin B ₁)	1.81 - 7.22	3	388 608	403 1.221	4.03	
	<i>Purine derivatives</i>						
50	Adenine sulf	0.062- 0.247 (0.062- 6.18)	6	456 — 858	421 1.245	139.0	3.09 mM 25% of the rats dead af- ter three days
51	Xanthine	4.93 - 16.44	2	127 935	422 1.208	0.1	
	<i>Cyanuric acid deriva- tives</i>						
52	Cyanuric acid	2.91 - 9.68	4	247 756	460 1.152	1.7	
53	Trimethyl-n- cyanurate	(0.18 - 2.92)	4			0	depressant
54	Trimethyl iso- cyanurate	0.73 - 2.19 (0.73 - 7.31)	3	192 111	286 1.179	11.0	3.66 mM depres- sant
55	Ammelide	(1.95 - 19.52)	3			0	
56	Ammeline	(3.93 - 19.66)	2			0	
57	Melamine	0.1 - 1.0 (0.1 - 20.0)	9	4023 — 6627	4203 1.213	76.5	>1.0 mM 140- 160% of fluid fed excreted, crystal- luria
58	Formoguanamine	0.023- 0.09 (0.023- 2.23)	6	362 — 1.346	388 1.232	347.1	
59	Acetoguanamine	1.0 - 5.0 (0.2 - 10.0)	5	199 399	392 1.228	4.5	5.0 mM diarrhea
60	Carboxy aceto- guanamine	(1.85 - 11.83)	2			0	

TABLE 1—*Concluded*

NO.	SUBSTANCE	USEFUL DIURETIC DOSE RANGE, (TOTAL DOSE RANGE TESTED)	NO. OF EXPTS. UTILIZED FOR COMPARISON	MEAN LOG ACTION \bar{y} MEAN LOG DOSE \bar{x}		ACTIVITY OF SUB- STANCE	OBSERVATIONS ABOUT MODE OF ACTION AT mM./kg.
				Sub- stance	Urea		
		mM./kg.					
61	Levulinoguan- amine	(3.45)	1			0	
62	α -Furoguanamine	(0.107- 5.65)	5			0	depressant, diar- rhea
63	α -Furoacrylo- guanamine	0.62 - 1.23 (0.62 - 6.16)	4	.234 -.030	.350 1.208	11.1	>1.23 mM: lethal within several days
64	4-Aminobenzo- guanamine	2.46 - 4.92	1	.241 .542	.257 1.169	3.5	
65	2-Phenylcincho- noganamine	(1.99 - 3.98)	1			0	
66	Acetoguanide	(4.96 - 19.84)	3			0	
67	Aminoacetoguan- amine	4.46 - 10.71	2	.272 .840	.360 1.169	1.7	
	<i>Varia:</i>						
68	Allantoin	(7.91 - 36.64)	2			0	
69	Urazol	(7.43 - 24.75)	2			0	
70	Barbituric acid	(9.76 - 19.51)	2			0	diarrhea

* All substances tested were chemically pure agreeing in analytic and physical constants with data in the literature.

† 0 means: doubtful or insignificant diuretic activity.

Melamine alone, among the highly active compounds, was found to represent the urea-type of diuretic action in that it has linear dose-action curve which begins to flatten only at levels of excretion considerably greater than 100 per cent of the administered water. No side actions or decline of the curve by overdosage are observed. On the other hand, adenine and formoguanamine, with activities greater than melamine, are not able to drain tissue-water from the normal rat. They, therefore, are similar to theobromine in their diuretic action. Besides these, a considerable number of substances were found which have biphasic dose-action curves, due to side-actions. Examples of this type are the higher amides, furo-compounds, amidines, and aminourea derivatives.

Still another factor may be involved in the evaluation of a substance as a diuretic, namely the time factor. The potency of tetrahydrofuroamide was found to change with the duration of the diuretic experiments. When the urinary excretion was measured as usual at the end of 5 hours the activity of tetrahydrofuroamide was found to be 1.2. But it was apparent from the rate of excretion at the end of the period that its diuretic action would be prolonged over five hours in contrast to urea and most of the other substances. Consequently, when the urinary excretion was measured at the end of eight hours, its potency was found to be 2.2, and at the end of 22 hours, 3.5.

TABLE 2a
35 Acetamidine HCl

EX PERIM DATE	CONTROL EX CRETION	LOG ACTIONS OF LOG (mM / kg) DOSES					b		ACTIVITY
		Acetamidine		Urea			Acetamid ne	Urea	
		121	422	1 097	1 319	1 398			
1947	per cent								
8-5	65 1	— 097	097	— 046		152	6445	6578	7 04
8-6	24 0	152	316	243		464	5449	7342	6 24
8-10	24 1	294	462	246	446		5582	9009	9 23
8-11	25 1	267	373	330	493		3522	7342	5 82
1943									
9-16	20 3	257	261	222		560	0133	1 1220	10 2
9-17	22 3	038	289	237		561	8339	1 0764	5 43
9-21	16 8	273	381	425		640	3588	7143	4 20
9-24	17 0	417	481	156*	439†		2126	9402	10 5
mean							4308 ± 0022	8656 ± 0079	
\bar{x}							2715	1 216	
\bar{y}							2663	348	
n							8	8	

* log dose = 0.021

† log dose = 1.222

Since the slopes are not parallel, the activities are not equal at different levels of effect. Computed for $\gamma = 30715$, the average log response of all observations, mean activity = 6.5

TABLE 2b
50 Adenine Sulfate

EXPERIM DATE	CONTROL EXCRETION	LOG ACTIONS OF LOG (mM/kg) DOSES						
		Adenine sulfate			Urea			
		-1 209	- 905	- 607	1 097	1 222	1 319	1 398
	per cent							
6/1/42	18.2		491	747	352		600	
6/2/42	11.0		378	575		303		452
6/3/42	23.6	220	406		265		479	
9/8/43	26.0	125	326		250			466
9/10	15.4		404	715	415			721
9/14	14.1		345	645	277			476

No significant variation in slope between urea and adenine sulfate was found. Hence using the combined slope, $b_c = 857 \pm 0.050$, $\bar{N} = 2.143 \pm 0.34$, and mean activity 139 ± 11 .

DISCUSSION The experimental results obtained in this and the previous paper on normal rats can be presented in three different ways. 1) The regression line is obtained by plotting log action as a function of log dose. Thus the diuretic

TABLE 2c
57. Melamine

EXPERIM. DATE	CONTROL EXCRETION	LOG ACTIONS OF LOG (mM./kg.) DOSES						
		Melamine				Urea		
		-.827	-.703	-.526	-.402	1.097	1.319	1.398
1942	<i>per cent</i>							
4-16	15.5		.560		.706	.458	.625	
8-13	28.6	.217		.338		.158		.413
8-14	19.5	.382		.539		.324		.607
1943								
8-17	27.4	.243		.377		.114		.466
8-18	21.8	.193		.426		.340		.560
8-19	24.7	.097		.407		.266		.468
8-20	18.7	.372		.571		.241		.548
8-25	15.9	.350		.688		.421		.670
8-26	19.5	.156		.620		.344		.542

No significant variation in slope between urea and melamine was found. Hence using the combined slope, $b_c = .813 \pm .090$, $\bar{M} = 1.884 \pm .027$ and mean activity = 76.5 ± 4.8 .

TABLE 2d
58. Formoguanamine

DATE	CONTROL EXCRETION	LOG ACTIONS OF LOG (mM./kg.) DOSES							
		Formoguanamine			Urea				
		-1.647	-1.340	-1.045	1.018	1.097	1.222	1.319	1.398
	<i>per cent</i>								
6/10/42	19.0		.450	.772			.382		.508
4/19/43	22.9	.281	.329			.368			.555
4/21/43	44.2	-.331	.175		.004			.210	
4/22/43	32.5	.042	.315		.212			.341	
4/23/43	26.1		.400	.547		.346			.521
4/26/43	17.4		.645	.721		.503			.701

No significant variation in slope between urea and Formoguanamine was found. Hence using the combined slope, $b_c = .686 \pm .123$, $\bar{M} = 2.5405 \pm .0472$, and mean activity = 347.1 ± 37.7 .

potency of the substance and the slope of its regression line are determined. The diuretic potency is merely the ratio of the millimoles of urea to those of the substance producing the same urinary excretion as urea. The slope and a change in the slope of the lines may give some indication of similarities in action. The regression lines of most of the substances tested are parallel to that of urea; but there was found one substance, sodium acetate, with a significantly steeper slope, and another, acetamidine hydrochloride, with a significantly flatter slope (fig. 1). In contrast to such substances as urea, melamine, adenine and formoguanamine there were found a number of substances with complex dose-action curves de-

creasing at "overdoses." Among this type of substances must be listed theophylline and caffeine. Such a curve indicates that the particular substance is toxic, because the excretion of urine is lessened either directly (Hg, Bi), or indirectly.

2) If the urinary excretion in percent of administered fluid is plotted as a function of log dose, two types of diuretics can be discerned. The first type when used in reasonably high doses removes as urine more than 100 per cent and up to 200 per cent, of the water fed. This means that tissue fluid can be drained by such substances as urea, lactamide, melamine and silyrgan (fig. 2). In contrast

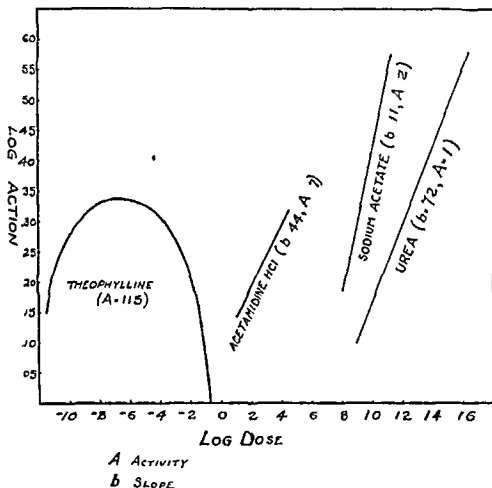


FIG. 1

to this, the second type of substance, although producing a considerable diuresis, (i.e. an accelerated urinary excretion as compared with control animals) is able only to remove excess tissue water from the rats even in high, non toxic doses. So the urinary excretion does not exceed 100 per cent of the water fed. Among the diuretics in common use theobromine was found to represent this type, and among the new substances studied formoguanamine and adenine sulfate.

As mentioned already in the previous paper, we assume a physiologically limiting factor rather than the chemical properties of the particular substances is responsible for this phenomenon.

3) When urinary excretion is plotted against time after feeding most of the substances tested show only minor differences from each other and from urea in latent period and the steepness of the curve. Minor differences are apparent

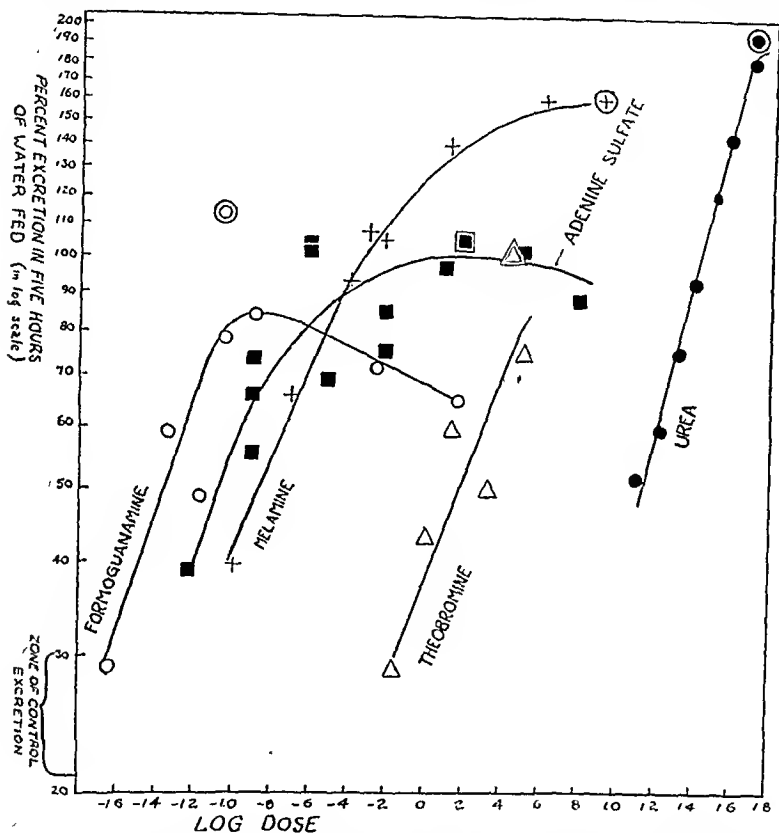
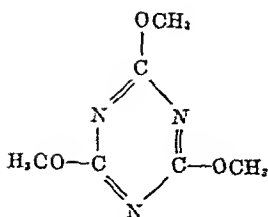
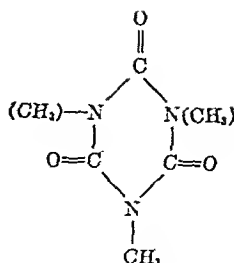


FIG. 2. PER CENT EXCRETION OF WATER ADMINISTERED, IN 5 HOURS BY VARIOUS DIURETICS
 ● mean values for urea, ⊙ highest individual value obtained.
 △ mean values for theobromine, ⊕ highest individual value obtained in 11 experiments.
 + mean values for melamine, ⊕ highest individual value obtained.
 ○ mean values for formoguanamine, ⊙ highest individual value obtained in 12 experiments.
 ■ individual values for adenine sulfate, ⊕ highest individual value obtained in 7 experiments.

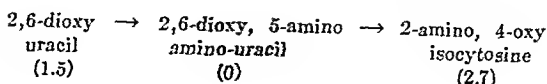
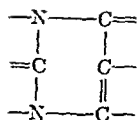
due to the somewhat different speeds of absorption. In three instances, however, namely salyrgan, bismuth sodium tartrate and tetrahydrofuroamide, the period of the diuretic action of the substance was considerably prolonged. There is some doubt in the case of the metal-organic substances whether this is due



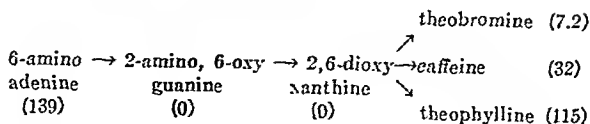
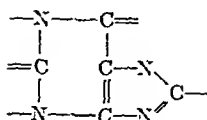
n-trimethylcyanurate
(0)



trimethyl-isocyanurate
(11.0)



2-methyl, 5 - R, 6-amino
thiamine HCl (Vitamin B₁) (4.1)



From this report it can be concluded that urca, the known xanthine diuretics, melamine, adenine and formoguanamine a.s.o. are representatives of one group of pharmaca containing the chemical group $\text{=N}-\text{C}-\text{N=}$ and characterized by diuretic action.¹

SUMMARY

1. Seventy amides, amines, amidines, urea, pyrimidine and purine derivatives and amino derivatives of the cyanuric acid were tested by the rat assay method for diuretic action. Melamine, adenine and formoguanamine were found to be potent diuretics.

2. The dependency of diuretic activity upon chemical structure was discussed, and some view-points of diuretic action presented.

The authors wish to thank Dr. C. I. Bliss for his advice and help in the statistical analysis of the data.

REFERENCES

- (1) LIPSCHITZ, HADIBIAN AND KERPCSAR; THIS JOURNAL, 79 (2), 97, 1943.

¹ Melamine, shown to possess diuretic activity by the rat assay method, has also been found to be diuretically active when tried in dog and man. The dosage of melamine required for diuresis in man has varied from 0.6 gm. to 3 gm. per day.

CARDIAC AND BLOOD PRESSURE EFFECTS OF PITOCIN (OXYTOCIN) IN MAN¹

R. A. WOODBURY, W. F. HAMILTON, PERRY P. VOLPITTO, B. E. ABREU AND
H. T. HARPER, Jr.

*From the Departments of Pharmacology, Physiology, Anesthesiology and Medicine,
University of Georgia School of Medicine, Augusta, Ga.*

Received for publication January 4, 1944

Recently (1) it was reported that in man, administrations of posterior pituitary preparations which contain the *oxytocic fraction* produce a transient but pronounced reduction in the arterial pressure. Proof was presented many years ago (2, 3) that this is true in the fowl. Yet, investigations of possible cardiovascular actions of oxytocin in man have been neglected probably because such a response is absent or very small in the mammals generally studied in the laboratory, i.e. dogs and cats. Vasodilation of cerebral vessels (4, 5) and marked reduction in the arterial pressure (5) have been observed in man following the administration of posterior pituitary extracts, but the importance of oxytocin in these responses was not determined.

The cardiovascular changes in mammals produced by pituitary preparations are not present attributed to the vasopressor principle (6). Only three reports have been found which indicate that the oxytocic fraction has any influence upon the mammalian cardiovascular system. Gaddum (7) stated that oxytocin caused a transient reduction in the blood pressure of some cats. Stehle (8) from studies on dogs believes that pressor and depressor actions may be inherent properties of the oxytocic molecule. Melville (9) observed in dogs that the oxytocic fraction antagonized but did not abolish the cardiac effects of the pressor principle.

The depressor effect observed in the fowl is reported to result from peripheral vasodilation (2, 10) and to be limited by the simultaneous powerful stimulating effect of posterior pituitary preparations upon the auricles and ventricles (2). Previous administration of atropine to the bird prevented the increase in ventricular amplitude (2) but did not abolish the fall of blood pressure (2, 10). Apparently acetylcholine activity is responsible for the cardiac effects. It has been suggested and disproved (10) that acetylcholine or histamine are responsible for the reduction in the blood pressure.

In the present study with 33 patients the mechanism of the fall in arterial pressure has been investigated in man. Arterial and venous pressure studies were accomplished using the hypodermic manometer (11, 12). In a few patients electrocardiograms, finger volume changes and arterial pressure pulses were recorded simultaneously.

RESULTS AND DISCUSSION. "Pituitin" injected intravenously in 3 units doses or into the wall of the uterus in 10 units doses caused a transient reduction in

¹ Aid from a grant from Eli Lilly and Company is gratefully acknowledged.

the arterial pressure of 30 to 50 mm. Hg. Within 2 to 5 minutes after the injection the blood pressure had returned to the pre-injection level. As shown in figs. 1 and 2, this reduction in blood pressure was produced by "pitocin" using 3 units of oxytocic activity, but was not produced by "pitressin" using 0.3 or even 3 units of vasopressor activity. These data, repeatedly observed from 28 patients, prove that this depressor action was related to the oxytocic fraction of the posterior pituitary preparation, rather than to the vasopressor fraction. This depressor action was obtained in males and in non-pregnant and in 3 and 6 months pregnant females. In three patients intramuscular injections of 10 oxytocic units lowered the arterial pressure 5 to 10 mm. Hg for periods beginning about 1 minute after the injection and continuing 5 minutes. Failure of earlier workers (13) to observe this depressor effect in man can be ascribed to their failure to measure the arterial pressure immediately after the injection.

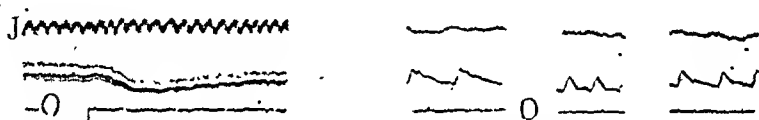


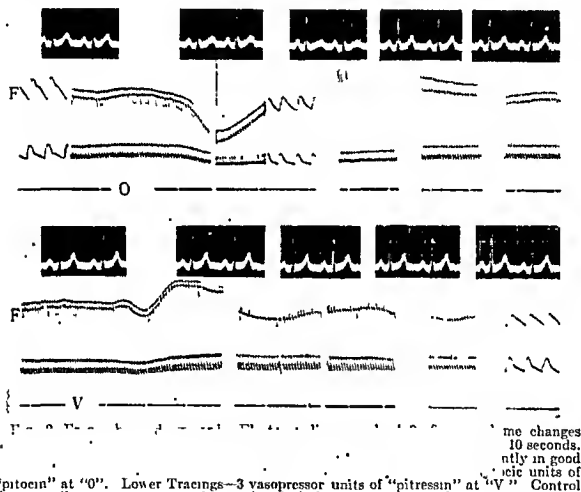
FIG. 1. From above downwards: Venous pressure pulses from the internal jugular, brachial arterial pressure pulses and base line interrupted at intervals of 10 seconds. *Left* tracings are from a female patient age 48, weight 58 kgm. Intravenous injection of 3 oxytocic units of "pitocin" at "0" lowered the arterial pressure within 20 seconds from 175/110 to 125/80 mm. Hg, and raised the venous pressure 1 or 2 mm. Hg. One minute later the arterial and venous pressures had nearly returned to the pre-injection level. *Right* tracings are from a male patient age 36, weight 68 kgm. The camera was operated with the photosensitive paper moving rapidly. This "spread out" the pressure pulses so that the contours could be investigated. The records in the figure were obtained immediately before, thirty seconds after and two minutes after the intravenous injection of 3 oxytocic units of "pitocin." While the arterial pressure was reduced from 140/85 to 115/68 mm. Hg, the pressure pulse contours (middle tracing) showed none of the characteristic changes which are associated with vasodilation, see text and compare with fig. 3.

Superficial investigations of the relationship between the amount of the effect and the size of the dose suggest that through the dose range from 1 to 4 units the relationship is fairly linear. In two patients intravenous administrations of 10 oxytocic units of "pitocin" elicited responses which were approximately 10 per cent greater and longer than those produced by injection of 4 units into these patients. Apparently intravenous doses of 10 units produce effects corresponding to the upper flat part of the usual parabolic effect dose curve (14). Slight tachyphylaxis was observed in man from four intravenous injections of 3 units of "pitocin" at 10 minute intervals. However, as in the fowl (10) and in the rabbit (15) larger doses might produce marked tachyphylaxis.

In the absence of anesthesia cardiac acceleration accompanied the low blood pressure. This acceleration, however, could be psychic in origin since "pitocin" did not modify the cardiac rate in the presence of ether anesthesia (3 patients) and cyclopropane anesthesia (7 patients). The blood pressure response to oxytocin was not modified by the previous administration of 2.5 mgm. of atropine sulfate though of course the heart was accelerated. During the period of the

low blood pressure the internal jugular pressure increased 1 or 2 mm. Hg (fig. 1). This agrees with earlier observations (5) and could have resulted either from an increased venous return secondary to peripheral vasodilation or from back pressure arising from decreased cardiac output. Both possibilities have been investigated.

Proof that peripheral vasodilation is not responsible for the low blood pressure in man has been obtained from pulse contour studies and from finger volume



pressure pulses so that the contours could be investigated, see text and compare with Fig. 3.

tracings. Histamine or amyl nitrite, drugs known to cause vasodilation, produce changes in the pulse contours which are characteristic of vasodilation (16) (fig. 3). These are 1) for any given pressure on the diastolic portion of the contour the rate of pressure descent is increased, 2) the pulse becomes more central in type, 3) standing waves are less pronounced and 4) the anacrotic notch appears or becomes more pronounced. These pulse contour changes which are characteristic of vasodilation (fig. 3) were not present after the injection of either "pituitrin" or "pitocin" (fig. 1, 2). Arterial pressure and finger volume tracings were

recorded simultaneously. These records did show some *delayed* increase in finger volume and finger pulse, which are evidences of vasodilation. However, these only appeared 90 to 120 seconds after the maximal fall of arterial pressure (fig. 2). Intravenous injections of pituitary preparations which contain the oxytocic principle bring about some vasodilation in man as in the fowl but the reduction in the arterial pressure in man precedes and is independent of any vasodilation.

Additional proof of the insignificant role of vasodilation was obtained from a patient with complete coarctation of the aorta where the arterial "Windkessel" is divided into two segments, one above and one below the coarctation. Earlier studies (17) have shown that vasodilator drugs increase the collateral circulation and reduce the pulse transmission time around the coarctation thereby lowering

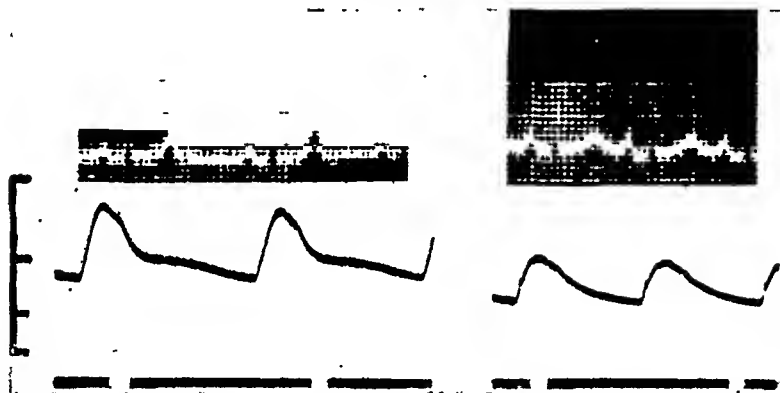


FIG. 3. Electrocardiograms lead 2, brachial arterial pressure pulses and base line interrupted at intervals of 1 second. Blood pressure scale is shown in units of 50 mm. Hg. Tracings were obtained from a male patient age 32, weight 74 kgm. At the break in the records 0.005 mgm. histamine phosphate was injected intravenously and 30 seconds of the record has been deleted. Histamine which is known to cause peripheral vasodilation produces pulse contour changes which are characteristic of peripheral vasodilation: 1) for any given pressure on the diastolic portion of the contour the rate of pressure descent is increased, 2) the pulse becomes more central in type, 3) standing waves are less pronounced and 4) the anaerotic notch appears or becomes more pronounced.

the blood pressure in the upper segment and raising it in the lower one. As shown in fig. 4, the intravenous injection of 3 oxytocic units of "pitocin" did not produce these changes but did markedly reduce the arterial pressures and pulse pressures above and below the coarctation. It also doubled the pulse transmission time around the coarctation. These changes can be caused only by a marked decrease in the cardiac beat and minute output. In the presence of normal or low arterial pressure decreased cardiac output only can result either from decreased venous return or from feeble cardiac contractions. Venous return was apparently adequate (see venous pressures fig. 1). These data prove that in man intravenous injections of "pitocin" *markedly weaken* cardiac contractions.

In 3 of the 4 patients studied, the electrocardiograms (fig. 2) show flattening of the T wave and a shortening of the iso-electric period throughout the period

of the low blood pressure. These are characteristic of hypoxia and anoxia (18) and could conceivably result from a reduced coronary blood flow. Yet the fact that these electrocardiographic changes are pronounced as soon as the blood pressure starts downwards indicates that they are not manifestations of any reduced coronary blood flow secondary to the low blood pressure. Coronary constriction probably does not occur since "pitocin" produces a transient pronounced fall in the arterial pressure of rabbits and it does not cause coronary vasoconstriction but actually causes slight coronary vasodilation in perfused rabbit hearts (19). The electrocardiographic changes produced by "pitocin" resemble those produced by histamine. Yet, the weak cardiac contractions and the insignificant amount of vasodilation present after the injections of "pitocin" are not characteristic effects of histamine (see above).

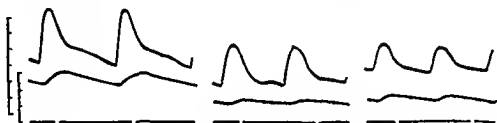


FIG. 4 Reproductions of brachial and femoral arterial pressure pulses secured from a rabbit. The vertical scale bars are shown in units of 25 mm. Hg. At the left are shown the pulse contours obtained before the injection of pitocin. At the right are shown the pulse contours obtained 24 minutes after the injection. Thirty seconds after the injection, the pulse contours are shown in the middle panel.

The electrocardiographic changes produced by "pitocin" along with the feeble cardiac contractions, the low blood pressure and small pulse pressure are typical of anoxia and might be manifestations of an interference with oxidative processes of the heart. This possibility is not in disagreement with the observation (20) that in dogs "pitocin" causes little or no interference with oxidative processes. Absence of this effect in dogs does not prove its absence in cardiac tissue in man since in dogs "pitocin" produces little or none of the cardiac and blood pressure changes which were observed in man.

The "pitocin" effects in man differ from those reported in the fowl. In man it is cardiac in origin while in the fowl vasodilation is responsible. In addition to this, man reacts to a much smaller injection and no definite acetylcholine activity of oxytocin could be demonstrated. The absence of appreciable acetylcholine effects in man could result from the extremely rapid rate of acetylcholine destruction in man (21).

These studies do not disagree with the fact that the vasopressor principle of posterior pituitary preparations has cardiovascular actions. They show that the

oxytocic principle also has cardiovascular actions in man. Of course "pitocin" is not a solution of a pure chemical compound. Yet, these cardiac effects are associated with the oxytocic fraction of pituitary preparations and may well be caused by the oxytocic substance or substances.

CONCLUSIONS

The species variation of the action of the oxytocic principle of posterior pituitary preparations is pronounced.

In man, pituitary preparations which contain the oxytocic fraction, transiently reduce the arterial pressure, weaken cardiac contractions and usually influence the electrocardiogram. These changes are not caused by either histamine or acetylcholine and apparently do not originate from a reduced coronary flow. Vasodilation sometimes also occurs, but it is independent of and is not responsible for the cardiac and the immediate blood pressure changes.

REFERENCES

- (1) WOODBURY, R. A., W. F. HAMILTON, AND P. P. VOLPITTO, *Am. Jour. Physiol.*, **129**: 500, 1940.
- (2) PATON, N., AND A. WATSON, *J. Physiol.*, **44**: 413, 1912.
- (3) HOGGEN, L. T., *Quart. Jour. Exper. Physiol.*, **15**: 155, 1925.
- (4) RAPHAEL, T., AND J. M. STANTON, *Arch. Neurol. and Psychiat.*, **2**: 389, 1919.
- (5) LOMAN, J., AND A. MYERSON, *Arch. Neurol. and Psychiat.*, **27**: 1226, 1932.
- (6) GOODMAN, L., AND A. GILMAN, *Pharmacological Basis of Therapeutics*. Macmillan Company, New York, 1941.
- (7) GADDUM, J. H., *J. Physiol.*, **65**: 434, 1928.
- (8) STEHLE, R. L., *J. Biol. Chem.*, **102**: 573, 1933.
- (9) MELVILLE, K. I., *J. A. M. A.*, **106**: 102, 1936.
- (10) MORASH, R., AND O. S. GIBBS, *THIS JOURNAL*, **37**: 475, 1929.
- (11) HAMILTON, W. F., G. BREWER AND I. BROTMAN, *Am. J. Physiol.*, **107**: 427, 1934.
- (12) HAMILTON, W. F., R. A. WOODBURY AND H. T. HARPER, JR., *J. A. M. A.*, **107**: 853, 1936.
- (13) GROLLMAN, A., AND E. M. K. GEILING, *THIS JOURNAL*, **46**: 447, 1932.
- (14) CLARK, A. J., *The Mode of Action of Drugs on Cells*. Williams & Wilkins, Baltimore, 1933.
- (15) WOODBURY, R. A., AND W. F. HAMILTON, unpublished observations.
- (16) HAMILTON, W. F., AND P. DOW, *Am. J. Physiol.*, in press.
- (17) WOODBURY, R. A., E. E. MURPHY AND W. F. HAMILTON, *Arch. Int. Med.*, **65**: 752, 1940.
- (18) ASHMAN, R., AND E. HULL, *Essentials of Electrocardiography*. Macmillan Company, New York, 1941.
- (19) WOODBURY, R. A., AND W. F. HAMILTON, *Fed. Proc.*, **1**: 172, 1942.
- (20) GEILING, E. M. K., AND A. M. DELAWDER, *Bul. Johns Hopkins Hosp.*, **51**: 1 and 335, 1932.
- (21) WEISS, S. AND L. B. ELLIS, *THIS JOURNAL*, **52**: 113, 1934.

TREATMENT OF EXPERIMENTAL RENAL HYPERTENSION WITH RENAL EXTRACTS¹

G E WAKERLIN, C A JOHNSON W G MOSS AND M L GOLDBERG

From the Departments of Physiology and Physiological Chemistry of the University of Illinois College of Medicine

Received for publication January 12 1944

We have already reported that daily intramuscular injections for four months or more of partially purified hog renal extract containing renin (in doses of 1 gm of fresh renal cortex equivalent per kg of body weight) produced striking reductions in the blood pressures of renal hypertensive dogs, whereas partially purified heat inactivated hog renin and partially purified dog renal extract containing renin each in 1 gm doses were without antihypertensive effects (1, 2) We pointed out that these therapeutic effects might be due to some type of immune response to heterologous hog renin or to the antihypertensive action of some substance in the non renin fraction of the partially purified hog renin solution We also indicated that antirenin was most probably not involved in the antihypertensive mechanism although this possibility, with reservations, was originally suggested by us (3)

In order to determine whether the antihypertensive potency of partially purified hog renin is due to renin or the non renin fraction, we have compared the therapeutic effects of highly purified hog renin with partially purified hog renin in renal hypertensive dogs In order to obtain further information concerning the heat lability of the active principle and the influence of homologous versus heterologous renins respectively we have also studied partially purified heat inactivated hog renin and partially purified dog renin in larger doses than previously employed by us Renal hypertensive dogs were also treated with partially purified hog liver extract prepared after the manner of renin to control the possibility of a non specific, foreign protein factor Antirenin studies were made to clarify further the relation of this antibody (antienzyme or antihormone) to the mechanism of the antihypertensive effect of partially purified hog renin

METHODS The methods used were in general similar to those previously employed by us Mean blood pressure readings were obtained by puncture of a femoral artery two or three times a week Blood urea nitrogen determinations urinalyses, and determinations of the body weight were made at monthly or bimonthly intervals and more frequently when indicated The hypertensive dogs employed in this study were subjected to a normotensive control interval of two months prior to constriction of the renal arteries followed by a minimum period of four months for stabilization of the hypertension The partially purified renal and liver extracts were prepared essentially by the method of Grossman (4), except that cold acetone instead of alcohol was employed as a dehydrating agent and much of the associated protein was removed by isoelectric precipitation Partially purified hog renin

¹ This work was aided by grants from the John and Mary R Markle Foundation Parke Davis and Company and the Graduate School Research Fund of the University of Illinois

was inactivated by heating at 70°C. for one-half hour. So-called highly purified hog renin² was prepared from partially purified hog renin by an ammonium sulphate precipitation which enabled removal of 84 per cent of the non-renin substances without appreciably affecting the amount of renin pressor activity. The partially purified renal and liver extracts were equivalent to 2 gm. of fresh tissue per cc. of solution and the highly purified renal extract to 5 gm. of fresh tissue per cc. The solutions were kept at 4°C. and preserved with 0.5 per cent phenol.

Treatment consisted of daily intramuscular injections for a period of six months. Five renal hypertensive dogs were treated with highly purified hog renin in a 3 gm. dose of fresh renal cortex equivalent per kg. of body weight and five other dogs with a 1 gm. dose. Three hypertensive dogs were treated with partially purified heat-inactivated hog renin in a 3 gm. dose; four dogs with a 3 gm. dose of partially purified dog renin; and three dogs with hog liver extract (prepared after the manner of partially purified renin)³ in a 3 gm. dose.

Blood serums were examined for antirenin before treatment, semimonthly during treatment and monthly or bimonthly after treatment. The technique consisted essentially in mixing the test serum with partially purified renin (1 cc. equivalent to 1 gm. of fresh cortex) and assaying immediately or shortly after by intravenous injection of the mixture into the etherized, nephrectomized dog. The presence of antirenin, of course, is demonstrated by partial or complete neutralization of the usual pressor effect of renin. The usual dose of renin solution was 0.25 cc. per kg. of assay animal. Antirenin titres were determined by using mixtures of serum and renin varying from 0.25:1 to 4:1, although a 2:1 volume ratio was most commonly employed. The serums tested for antirenin were suitably controlled with serums from untreated normotensive and untreated hypertensive dogs. Antirenin titres were regularly ascertained for dog renin and less frequently for hog renin.

RESULTS. 1. *Highly Purified Hog Renin.* (a) *3 gm. dose.* The first dog of this group with a pretreatment hypertensive range of 150–180 mm. Hg showed a gradual decrease in blood pressure during the six months' period of treatment until the precontraction normotensive level of 120–140 mm. Hg was reached during the last month of therapy. During fourteen months after treatment, the blood pressure has remained in the normotensive range with a slight tendency to rise (fig. 1). The second dog showed a reduction in blood pressure during the second and third months of treatment from a hypertensive level of 150–180 mm. Hg to the normotensive level of 120–140 mm. Hg. Three and one-half months after therapy the pressures were still in this range when the dog died of lobar pneumonia. The third, fourth, and fifth dogs showed blood pressure decreases during the second and third months of therapy from hypertensive levels of 170–190, 140–180, and 170–190 to ranges of 140–160, 120–150 and 140–170 which were 10–30 mm. Hg above the precontraction normotensive ranges of 130–150, 100–120, and 120–140 mm. Hg, respectively. No further reductions in pressure occurred during the last three months of treatment. The third dog died from an improperly administered anesthetic two months after therapy. The blood pressures of the remaining two dogs gradually returned to

² Highly purified hog renin was supplied through the courtesy of Dr. Oliver Kamm of Parke, Davis and Company.

³ In other words, minced hog liver (which, of course, is devoid of renin) was successively dehydrated with cold acetone and ether. An alkaline-saline extract of the resulting powder was brought to a pH of 4.7 and the precipitating proteins removed. The filtrate with 0.5% phenol added is hereinafter referred to as "hog liver extract" or "hog liver extract prepared after the manner of partially purified renin."

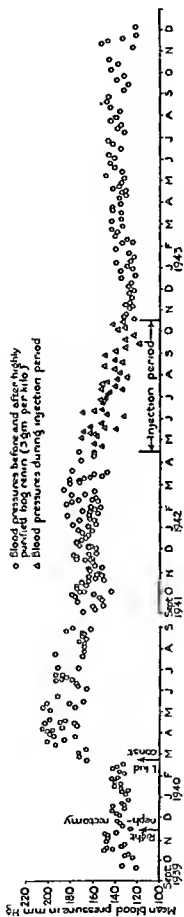


FIG. 1

the pretreatment hypertensive levels during the first five months following injections. The blood pressures of the fifth dog are recorded in fig. 2.

Antirenin became demonstrable in the serum of the first dog one month after the beginning of treatment and was still present in moderate (1:1) titer fourteen months after treatment was discontinued. Antirenin appeared in the serums of the other four dogs one, four, four, and one months respectively after the beginning of treatment and disappeared one, two, one, and two months following treatment.

(b) 1 gm. dose. The first dog of this group showed a hypertensive range of 150–180 mm. Hg which decreased gradually to a level of 130–150 during the first four months of treatment but not to the precontraction normotensive range

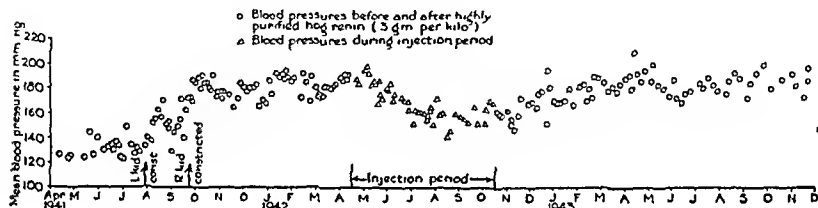


FIG. 2

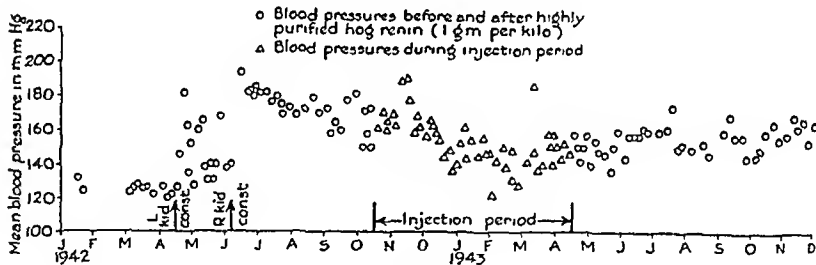


FIG. 3

of 120–130. Following therapy the pressures gradually rose to the pretreatment hypertensive range over a period of eight months (fig. 3). The second, third, fourth, and fifth dogs with original normotensive levels of 120–140 and hypertensive ranges of 140–180, 150–180, 160–190 and 150–180 showed no significant changes in blood pressure during treatment or for eight months thereafter. Fig. 4 which illustrates the blood pressure record of the fourth animal is typical.

Appearance and disappearance times for antirenin were not determined for this group. Quantitative antirenin assays during the fourth, fifth, and sixth months of treatment showed minimum complete neutralization ratios of 4:1, 0.25:1, 0.25:1, and 0.5:1 respectively for the first four dogs. Quantitative assays were not done on the fifth dog.

2. Partially Purified Heat-inactivated Hog Renin. The first and second dogs

of this group showed normotensive levels of 120-140 and 90-120 mm Hg and pretreatment hypertensive levels of 140-170. During the injection period there was a decrease in pressures to 110-140 and 120-140 respectively with a gradual return to near pretreatment hypertensive levels during the fourteen months following treatment. The results for the second dog are shown in fig 5. The third animal with a normotensive level of 120-140 showed no important change from its hypertensive level of 150-180 during treatment. The slightly lower pressures of this dog during the four months following treatment are not considered significant (fig 6).

None of these dogs developed antirenin during or after treatment.

3 Partially Purified Dog Renin The four dogs of this group showed normotensive levels of 110-130, 120-140, 120-150 and 120-140 mm Hg and hypertensive ranges of 140-170, 160-180, 190-230 and 140-180 respectively. There were no significant changes during treatment or during a fourteen months' period following treatment. Fig 7 for the fourth dog is more or less typical.

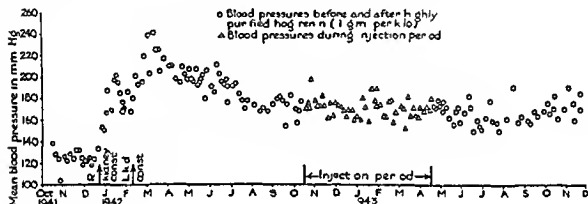


FIG 4

None of the dogs developed antirenin during or after treatment.

4 Hog Luer Extract Prepared after the Manner of Partially Purified Renin The three dogs comprising this group had normotensive levels of 110-130, 120-140, and 120-140 and hypertensive levels of 150-180, 140-190, and 140-180 mm Hg respectively. There were no significant changes in blood pressure during or after treatment as illustrated by fig 8 for the third animal.

None of the dogs of this group developed antirenin.

5 Toxicity of the Extracts Confirming our previous reports, none of the twenty dogs showed any evidence of local or general toxic effects from the extracts. Their appetites remained excellent, their body weights constant, and their temperatures, urine, and blood urea nitrogens normal throughout.

Discussion Table 1 summarizes the results reported here as well as previously reported pertinent findings. In the table the renin activities of the extracts are compared on an arbitrary scale, 1.0 denoting the renin activity of partially purified hog renin derived from 1 gm of fresh renal cortex. This is approximately equal to 1 Goldblatt unit (5). The non renin fraction of partially purified hog renin derived from 1 gm of cortex is also arbitrarily valued at 1.0.

for purposes of comparison. As indicated by the table the renin activity of partially purified dog renin is approximately equal to that of the corresponding hog renin as assayed on the dog. The amounts of renin and non-renin substances listed for highly purified hog renin result from the fact that, as already stated, the process of purification removed 84 per cent of the non-renin fraction without

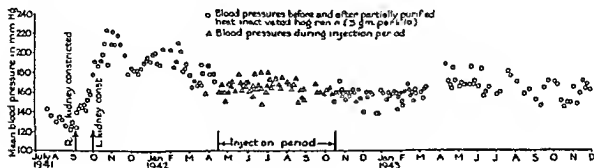


FIG. 6

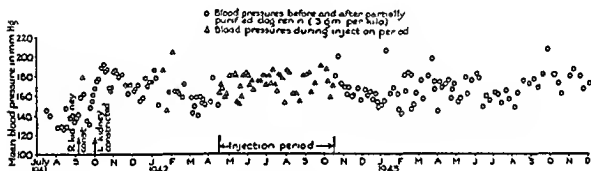


FIG. 7

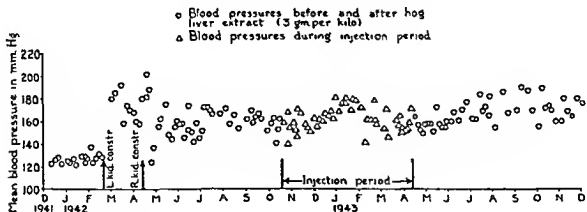


FIG. 8

significantly altering the renin activity. Heat inactivation of partially purified hog renin, of course, also resulted in some precipitation of the non-renin fraction. The hog liver extract showed a somewhat higher protein concentration (which is designated non-renin fraction in table 1) than the partially purified renins (1.7 per cent as compared with 1.0 per cent), calculating protein from N.

Table 1 shows no correlation between the antihypertensive effects and the renin activities of partially purified and highly purified hog renins. On the other hand, a relation between the values of the non-renin fractions and the anti-hypertensive effects is evident. This strongly suggests but does not prove that the antihypertensive effect of hog renal extracts containing renin is due to some principle in the non-renin fraction. Obviously there is also the possibility that a combination of renin and some principle in the non-renin fraction may be necessary.

Table 1 also indicates that the antihypertensive effect of partially purified hog renin is not completely destroyed by heating at 70°C. for one-half hour, since the 3 gm. dose showed fair antihypertensive activity. This result also suggests that the antihypertensive activity is in the non-renin fraction.

TABLE I
Treatment of experimental renal hypertension with renal extracts

EXTRACT	NO. OF HYPERTENSIVE DOGS	DOSE PER KG *	RENIN	NONRENIN FRACTION	ANTIHYPERTENSIVE EFFECT
		gm			
PPHog renin†	4	1	1.0	1.0	Excellent
HPHog renin	5	3	3.0	0.5	Fairly good
HPHog renin	5	1	1.0	0.16	Poor
PPHeat-inactivated hog renin†.	2	1	0.0	<1.0	None
PPHeat-inactivated hog renin.	3	3	0.0	<3.0	Fair
PPDog renin†	2	1	1.0	1.0	None
PPDog renin	4	3	3.0	3.0	None
Hog liver extract	3	3	0.0	>3.0	None

PP = partially purified, HP = highly purified.

* In terms of fresh renal cortex equivalent.

† Previously reported (1).

The lack of antihypertensive potency of partially purified dog renin is difficult to explain at present. Dog kidney may contain a lower concentration of antihypertensive principle. Or some type of immune response, other than antirenin or antihypertensin (6) (possibly to some constituent of the non-renin fraction) may be involved in the antihypertensive effect of the hog renal extracts. The results with hog liver extract, however, appear to rule out a foreign protein effect and suggest that the antihypertensive activity is specific for kidney.

The possible involvement of antirenin in the antihypertensive effect of our hog renal extracts is largely excluded, although Goldblatt (7) recently favored the antirenin hypothesis. With the exception of the first dog, the dogs treated with the 3 gm. dose of highly purified hog renin showed a poor correlation between the appearance and disappearance times of antirenin and the blood pressure reductions and subsequent returns to the pretreatment hypertensive levels. The

excellent correlation in the case of the first dog is most probably coincidental rather than causal. There was no correlation between the antirenin titers of the dogs treated with the 1 gm. dose of highly purified hog renin and the modest antihypertensive effect observed. Thus the only dog of this group to respond with a reduction in blood pressure evidenced the lowest antirenin titer whereas two of the other dogs which were therapeutic failures maintained relatively high titers. Moreover, partially purified heat-inactivated hog renin, although moderately antihypertensive, did not produce antirenin.

The relation of the active principle of our hog renal extracts to the antihypertensive activity reported by Page and his coworkers (8) and Grollman, Harrison and their colleagues (9) remains to be determined. Our extracts are effective in much smaller amounts of renal cortex equivalent and appear to be nontoxic.

Since the results reported here suggest that the antihypertensive effect is in the non-renin fraction, we are now studying the antihypertensive potency of various preparations of this fraction in renal hypertensive dogs.

CONCLUSIONS

1. The antihypertensive effect of partially purified hog renin in renal hypertensive dogs is definitely superior to highly purified hog renin, suggesting that the active principle is in the non-renin fraction.

2. Partially purified heat-inactivated hog renin possesses moderate antihypertensive activity, indicating that the active principle is partially heat-stable.

3. Partially purified dog renin is not antihypertensive in three times the effective dose of hog kidney, suggesting either that the concentration of the antihypertensive principle is considerably less in dog kidney or that some type of immune response not evoked by homologous renal extract is involved.

4. Hog liver extract prepared after the manner of partially purified renin was ineffective antihypertensively, suggesting that the antihypertensive potency of our hog renal extracts is not due to a foreign protein effect and that the potency is specific for kidney.

5. A role for antirenin in the antihypertensive mechanism is largely excluded.

6. A study of the antihypertensive potency of the non-renin fraction of partially purified hog renal extract containing renin is well-warranted and now under way.

We are grateful to R. E. Vessey and M. C. Tanner for technical assistance.

BIBLIOGRAPHY

- (1) WAKERLIN, G. E., AND JOHNSON, C. A.: The effect of renin on experimental renal hypertension in the dog. *J. A. M. A.*, 117: 416, 1941.
- (2) WAKERLIN, G. E., JOHNSON, C. A., SMITH, E. L., GOMBERG, B., WEIR, J. R., MOSS, W. G. AND GOLDBERG, M. L.: Treatment of experimental renal hypertension with partially purified renin. *Am. Ht. J.*, 25: 1, 1943.
- (3) WAKERLIN, G. E., JOHNSON, C. A., GOMBERG, B., AND GOLDBERG, M. L.: Reduction in the blood pressures of renal hypertensive dogs with hog renin. *Science*, 93: 332 1941.

- (4) GROSSMAN, E. B.: Preparation of extracts of the renal pressor substance. *Proc. Soc. Exp. Biol. & Med.*, **39**: 40, 1938.
- (5) GOLDBLATT, H., KATZ, Y. J., LEWIS, H. A., AND RICHARDSON, E.: Studies on experimental hypertension. XX. The bioassay of renin. *J. Exp. Med.*, **77**: 309, 1943.
- (6) GOLDBERG, M. L., AND WAKERLIN, G. E.: Unpublished data.
- (7) GOLDBLATT, H., KATZ, Y. J., LEWIS, H. A., RICHARDSON, E., GUEURA-ROJAS, A., AND GOLLAN, F.: On the nature and properties of antirenin. *Proc. Cen. Soc. Clin. Res.*, **15**: 31, 1942.
- (8) PAGE, I. H., HELMER, O. M., KOHLSTAEDT, K. G., KEMP, G. F., CORCORAN, A. C., AND TAYLOR, R. D.: A progress report of investigations concerned with the experimental treatment of hypertension with kidney extracts. *Ann. Int. Med.*, **18**: 29, 1943.
- (9) GROLLMAN, A., AND HARRISON, T. R.: Further studies on the separation from kidney tissue of a substance capable of reducing the blood pressure in experimentally induced hypertension. *J. Pharm. & Exp. Therap.*, **78**: 174, 1943.

THE ADRENOLYTIC AND SYMPATHOLYTIC ACTIONS OF YOHIMBINE AND ETHYL YOHIMBINE¹

FREDRICK F YONKMAN, DON STILWELL AND ROBERT JEREMIAS

Department of Pharmacology and Therapeutics, Wayne University College of Medicine, Detroit, Michigan

Received for publication January 25, 1944

Adrenolysis and sympatholysis are not synonymous despite the fact that both denote a loss of sympathetic or adrenergic like functions. It is conceivable that responses to epinephrine may be lost (1) under the influence of so-called 'sympathetic depressants' while reactions to faradization of sympathetic nerves may persist. Should this be the case under the influence of such an agent as the yohimbine radicle, adrenolysis would prevail in the absence of true sympatholysis. One might be tempted to call this pseudo sympatholysis.

Some time ago (2) (3) (4) statements were made in regard to the sympathetic depressant or antisymphatheticomimetic action of yohimbine compounds in relation to cervical sympathetic and vasoconstrictor neural reactions. The doses usually required for epinephrine reversal effects ranged between 2 and 8 mgm of ethyl yohimbine per kgm by the intravenous route and this range of dosage was also usually sufficient to nullify the effects of faradization of the cervical sympathetic nerve upon submaxillary salivation (4). At that time it was observed that cervical sympathetic paralysis of salivation was not always coincident with pupillary paralysis and with epinephrine reversal as demonstrated by blood pressure reactions. Therefore, our present experiments were designed to determine (a) whether a certain selectivity or specificity of action prevailed for the yohimbine radicle and if so, in what sequence certain sympathetic responses might be inhibited or erased, (b) the doses of yohimbine and ethyl yohimbine required for inhibition of various sympathetic reactions and (c) whether sympatholysis coexisted with adrenolysis.²

METHOD Twenty four cats were anesthetized with urethane gastrically and prepared for the insertion of cannulae into the trachea, femoral vein, carotid or femoral artery and Wharton's submaxillary duct. The vagi were sectioned and the cervical sympathetic and chorda tympani nerves were made accessible for faradization. The nictitating membrane was needled, threaded and attached for kymographic registration of its responses, and pupillary reactions were observed across a millimeter rule. Blood pressure and salivation were recorded manometrically. Thus, it was possible with these combined techniques to observe a general vascular response to epinephrine and the yohimbic radicle concomitantly.

¹ All references to yohimbine and ethyl yohimbine imply that the hydrochloride forms were employed. Yohimbine HCl was made available to us by Dr. D. F. Robertson of the Merck Company and ethyl yohimbine HCl represents an eight year old sample which was originally supplied to us by Hoffman-La Roche Company through courtesy of Drs. A. G. Young and D. Worral of Boston, Mass.

² We wish to acknowledge the cooperation in this project of Dr. Robert Byberg of Henry Ford Hospital and Dr. Donel Sullivan of Detroit Receiving Hospital, both former assistants in Pharmacology.

with three ipsilateral cervical sympathetic nerve functions, namely: salivation, mydriasis and retraction of the nictitating membrane.

In some experiments atropine sulphate and pilocarpine nitrate were employed to test cholinergically controlled salivation after inhibition of adrenergically controlled secretion by yohimbine and ethyl yohimbine. Pituitrin and angiotonin² were also administered to determine their effects upon these lytic states. All drugs were injected intravenously in physiologic sodium chloride solution and usually in variable amounts, the dosage depending upon the effect desired.

RESULTS. Not all of our experiments were satisfactory from the point of view of the successful study of all adrenergic and sympathetic functions involved in the same animal. Some of our early experiments did not include kymograms of the reactions of the nictitating membrane, and on occasion no salivary secretion resulted following epinephrine or faradization. In rare instances secretory responses were so minimal as to lend themselves with difficulty to proper interpretation in terms of accurate quantitation. However, in nineteen experiments sufficient evidence of sympathetic depression was obtained to warrant drawing

TABLE 1

Development of adrenolysis (A) and sympatholysis (S) by yohimbine HCl and ethyl yohimbine HCl in cats

	EPINEPH- RINE REVERSAL A	SALIVATION		MYDRIASIS		RETRACTION OF NIC- TITATING MEMBRANE	
		A	S	A	S	A	S
Yohimbine HCl.....	2-5*	2-5	4-5	5-6	28.3+	4-6	28.3
Ethyl yohimbine HCl.....	2-7	3-8	5-6	3	15+	3	15

* All doses expressed in mgm. per kgm.

conclusions regarding adrenolysis and sympatholysis. The ranges of effective doses of yohimbine and ethyl yohimbine are stated in mgm. per kgm. in table 1.

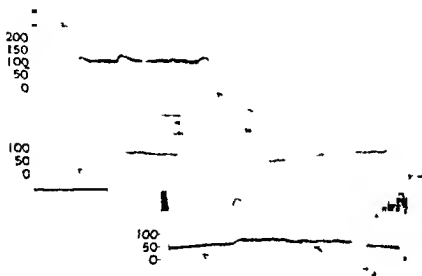
Circulatory manifestations of epinephrine-reversal were usually the first evidence of the adrenolytic action of yohimbine and ethyl yohimbine. This reversal resulted, as a rule, with 2 mgm./kgm. of yohimbine and with 3 mgm./kgm. of ethyl yohimbine. Almost simultaneously a depression of salivation occurred but occasionally slightly larger doses of both yohimbine (5 mgm./kgm.) and ethyl yohimbine (8 mgm./kgm.) were required to nullify epinephrine-induced salivation than were necessary for most vascular epinephrine-reversals. Mydriasis and retraction of the nictitating membrane almost invariably failed to occur under doses of epinephrine which had no effect on salivation. This was characteristic especially of ethyl yohimbine. Thus, it seemed that all adrenergic functions studied here which had responded to epinephrine were practically simultaneously inhibited by yohimbine and ethyl yohimbine. The smallest effective adrenolytic dose of both drugs was 2 mgm. per kgm. whereas the largest was 8 mgm. per kgm. A typical kymogram is presented in fig. 1.

Sympatholysis was invariably preceded by adrenolysis, the former occurring

²Supplied to us by courtesy of Dr. Irvine H. Page of the Lilly Laboratory for Clinical Research, Indianapolis, Indiana.

with somewhat higher doses of yohimbine and ethyl yohimbine. Salivation was the first cervical sympathetic function to be depressed, then retraction of the nictitating membrane and finally contraction of the dilator pupillae. The latter, however, was never completely obliterated.

The smallest sympatholytic doses of yohimbine and ethyl yohimbine for salivation were respectively, 4 and 5 mgm per kgm, for nictitating membrane responses, 28.3 and 15 mgm per kgm. Mydriasis was diminished by the latter doses but never abolished.



for 10 seconds

4 Atropine sulphate 0.4 mgm/kgm
5 13 16 26 Epinephrine HCl 0.02 mgm/kgm
6 to 9 11 22 Ethyl yohimbine HCl 3 mgm/kgm

24 Ethyl yohimbine, 6 mgm/kgm

27 Ethyl yohimbine 8.3 mgm/kgm

Record from 17 to 22 not included. During this interval 5 mgm of ethyl yohimbine HCl were injected and a clot was removed from the arterial cannula. Note that sympatholysis of salivation occurred at 12 and adrenolysis at 13. Not until 29, at a total dose of 8.3 mgm/kgm, did some sympatholytic reactions for the iris

Atropine, pilocarpine, angiotonin and pituitin did not interfere with the development of these lytic conditions.

DISCUSSION AND COMMENT Yohimbine and ethyl yohimbine not only nullify responses to epinephrine but also insulate against impulses initiated by faradization of sympathetic nerves. Our experiments indicate that adrenolysis occurs quite generally for the sympathin E complexes studied, whereas, sympatholysis appears successively in these same S E complexes in the order cited: S E mechanisms of the glandular type first, then those of the nictitating membrane and finally those associated with the dilator pupillae. We have also seen splanchnic reversal or sympatholysis after ethyl yohimbine (fig. 2) but it was not primarily

sought in these experiments. Recently Koppányi and his co-workers (5) reported that epinephrine-reversal as induced by yohimbine did not necessarily indicate that sympatholysis prevailed. In their hands splanchnic nerve faradization invariably produced an initial rise in tension. It is problematic what the lytic effect of larger doses of yohimbine might have been and an extended study of this type of vascular response seems warranted, especially with the use of large doses of ethyl yohimbine. It is evident, nevertheless, that complete lysis can be produced in the effector mechanisms associated with the cervical sympathetic nerve and it may well be that S-E complexes in the vascular bed

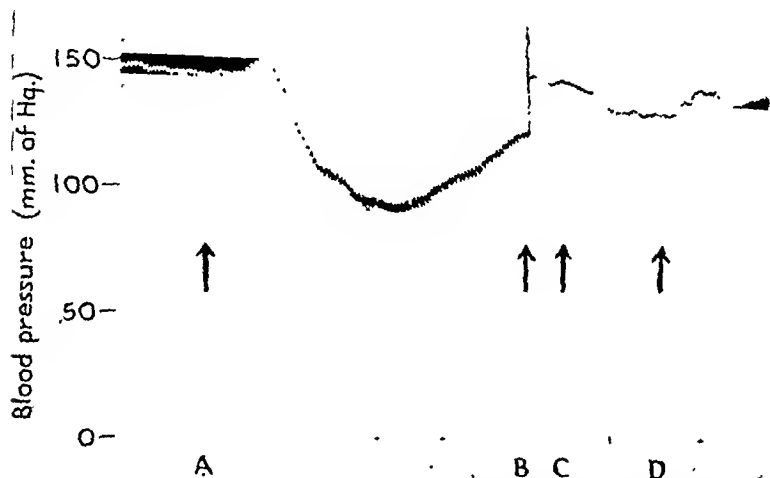


FIG. 2. Cat, 2.8 kgm. Feb. 2, 1942. Blood pressure under urethane anesthesia. Time in 5 second intervals. Prior to A drugs had been injected in the following order and dosage in terms of mgm. per kgm.: epinephrine, 0.01, ethyl yohimbine, 3.0, angiotonin, 0.02 cc. Adrenolysis prevailed at A and sympatholysis of vasoconstrictor components at C since vasodilation resulted from splanchnic stimulation.

A. Epinephrine, 0.01 mgm./kgm.

B. Interval of 4 minutes.

C. Faradization of splanchnic nerves begun.

D. Faradization of splanchnic nerves completed.

might prove to be as resistant to sympatholysis as are those of the iris and nictitating membrane.

As stated previously adrenolysis does not imply that sympatholysis prevails. However, the reverse is true; when the latter is established with the yohimbine radicle, the former condition also invariably prevails.

The conditions of lysis are not influenced by such drugs as atropine and pilocarpine. In order to obviate vagal reflexes associated with vascular tension rises as produced by epinephrine, the heart was insulated either by double vagotomy or the injection of atropine. In no instance did either of these measures prevent or seem to modify the establishment of adrenolysis or sympa-

tolysis Neither did these lytic conditions interfere with eholiacrgie secretion as produced by faradization of the ehorda tympani nerve or injection of pilo carpine Elsewhere (6) it has been demonstrated that myotropic drugs such as angiotonin and pituitrin also do not interfere with the development of either lytic state, likewise, these functional losses of epinephrine and sympathetic actions do not modify the contractile responses to such myotropic agents

To recapitulate, lysis, once effected by the yohimbine radicle, is resistant to the influences of other drugs such as atropine, angiotonin and pituitrin, and the ordinary effects of these agents are little, if at all, influenced by the preasea of lytic amouats of yohimbine and ethyl yohimbine

Since the yohimbine nucleus is so poteatly adrenolytic and, in amounts well tolerated by anesthetized animals is also sympatholytic, it becomes an excellat agent for the purpose of demoastrating adrenolysis and sympatholysis The results obtained in a comparative study of these lytic states as induced by yohimbine, ethyl yohimbine, ergotoxin and F 933 will be presented as a separate coatribution (7)

Ethyl yohimbine, which is less toxic than yohimbine (3) can well be thought of as the 'atropine' of the sympathetic nervous system especially in relation to augmentory types of adrenergic control The clinical value of this type of drug in certain conditions of hypersympatheticotonia is still a moot point

CONCLUSIONS

1 Yohimbine HCl and ethyl yohimbine HCl are adrenolytic for submaxillary salivation and vasomotor reversal in doses raaging from 2 to 7 mgm per kgm while larger doses are gencrally necessary for the suppression of the nictitating membrane and mydriatic responses to epinephrine

2 The yohimbine salts are sympatholytic for salivary reactions in higher doses than are required for adrenolysis ranging from 4 mgm per kgm for yohimbine to 6 mgm per kgm for etbyl yohimbine, doses for sympatholysis of nictitating membrane retraction range from 15 mgm per kgm for ethyl yohim bine to 28 mgm per kgm for yohimbine, and for sympatholysis of mydriatic action, doses are above 15 mgm per kgm for ethyl yohimbine and more than 28 mgm per kgm for yohimbine

3 In regard to the function of end organs supplied by eervial sympathetic nerve fibers adrenolysis invariably precedes sympatholysis

4 Adrenolysis and sympatholysis as produced by yohimbine HCl and ethyl yohimbine HCl are neither preveated nor significantly affected by such drugs as atropine pilocarpine and angiotonin Coaversely, the usual actions of these latter drugs are not significantly altered by the action of the former

REFERENCES

- (1) SACHS AND YONKMAN THIS JOURNAL 75 105 1942
- (2) HAMET Compt rend Acad d sc 180 2074 1925
- (3) YOUNG THIS JOURNAL 54 164 1935
- (4) YONKMAN AND YOUNG THIS JOURNAL 63 40 1938
- (5) KOPFANYI LINEGAR AND HERWICK Federation Proc 2 83 1943
- (6) YONKMAN JEREMIAS AND STILWELL Proc Soc Exp Biol & Med 54 204 1943
- (7) YONKMAN CHASE LEHMAN AND CRAVER To be published

TOXICOLOGICAL STUDIES OF PHTHALYLSULFATHIAZOLE¹

PAUL A. MATTIS, WILBUR M. BENSON AND ETHOL S. KOELLE

WITH THE TECHNICAL ASSISTANCE OF ETHEL WILLIAMS AND S. E. MCKINNEY

From the Pharmacological Laboratories, Research Division, Sharp and Dohme, Inc., Glenolden, Pa.

Received for publication February 4, 1944

A series of N⁴ dicarboxylic acid-substituted sulfonamides which resists absorption from the gastrointestinal tract was found to produce varying degrees of intestinal bacteriostasis (1). Toxicological studies of succinylsulfathiazole, one of this series, have been described previously (2). Another compound in this group, 2-(N⁴-phthalyl-sulfanilamido)-thiazole (phthalylsulfathiazole)² has recently engaged our attention. Preliminary reports (3, 4) indicated that this compound exerts a marked bacteriostatic effect on the intestinal coliform organisms of dogs, and that smaller doses are required to produce this effect than are necessary with succinylsulfathiazole.

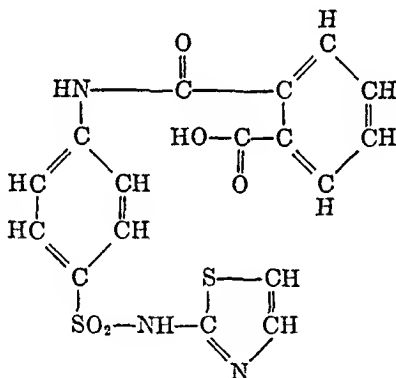


FIG. 1

In these experiments we have studied (a) the acute toxicity of phthalylsulfathiazole in mice, (b) the effects of parenteral administration of phthalylsulfathiazole in monkeys, and (c) the effects of prolonged oral administration of the

¹ Sharp and Dohme has applied its trade-mark 'Sulfathalidine' to this new sulfonamide.

² Phthalylsulfathiazole is a white crystalline powder, practically insoluble in water or weak acid. It is dissolved by an excess of an aqueous solution of sodium bicarbonate or carbonate at room temperature with the liberation of carbon dioxide. A 5 per cent solution of the sodium salt of phthalylsulfathiazole may be prepared by dissolving the drug in the appropriate volume of a 5 per cent solution of sodium bicarbonate. Twenty per cent solutions of the sodium salt of phthalylsulfathiazole are readily prepared with the aid of sodium hydroxide. Such solutions may be adjusted to a pH of about 7.6, but will deposit crystals on standing at room temperature.

drug to rats and monkeys. It was found that phthalylsulfathiazole could be administered orally to animals either in repeated doses or in massive single doses without producing any manifestations of toxicity. The sulfonamide concentration in the blood during such treatment remained low. Only by parenteral administration of phthalylsulfathiazole in doses sufficient to produce high concentrations of drug was it possible to produce signs of toxicity.

EXPERIMENTAL Acute Toxicity Oral Doses of 10 grams of phthalylsulfathiazole per kgm, in the form of a 25 per cent suspension in 0.5 per cent tragacanth, were administered orally to 24 female white mice (Carworth CF₁). The mean concentration in the blood (10 mice), two hours following gastric intubation, was 2.4 mgm per 100 cc of "free" and 1.7 mgm per 100 cc of "conjugated" sulfathiazole.^{3,4} No abnormalities of behavior or signs of toxicity were observed following the above dose.

Intraperitoneal toxicity (olive oil suspension) In order to secure data on the acute toxicity of this drug it was necessary to resort to a route of administration other than oral, namely, the injection of a suspension of the finely powdered drug in olive oil (25 cc per kgm) into the peritoneal cavity (6). The LD₅₀ (Behrens' method (7)) was found to be approximately 0.92 gram per kgm of body weight. Data from this experiment expressing percentage mortality and concentrations attained in the blood are shown in table 1.

Following intraperitoneal injection with the larger doses of phthalylsulfathiazole suspended in olive oil, the mice passed through a period of convulsive trembling, after which the animals became lethargic, some showed a loss of various voluntary muscular reflexes. Spasmodic convulsions also occurred, together with opisthotonus and erection of the tail in an occasional animal. Respiration became labored, lacrymation occurred, and the ears, tail and feet showed redness resembling peripheral vasodilatation. Heart action persisted after respiration had ceased.

Histopathological examination of tissues taken from mice dying six hours after intraperitoneal dosage in the range of the LD₅₀ to the LD₁₀₀, revealed lesions in the kidneys such as early degenerative changes of the tubular epithelium, granular and cellular debris, and presumptive evidence of crystals in the tubular lumina.

* The sulfathiazole liberated by acid hydrolysis from combined forms ("conjugated") is derived from both phthalylsulfathiazole and acetylsulfathiazole. The latter can be formed only from the small amounts of sulfathiazole released from phthalylsulfathiazole. It is probable, therefore, that "conjugated" sulfathiazole is composed essentially of phthalylsulfathiazole. Results in terms of sulfathiazole may be multiplied by 1.58 in order to express as phthalylsulfathiazole.

* The method of Bratton and Marshall (5) was employed in the determination of the "free" sulfathiazole present in the blood and urine. It is essential to carry out the determination of free sulfathiazole with a minimum of delay after p-toluenesulfonic acid has been added to precipitate the proteins and to give the acidity necessary for diazotization since in the presence of acid phthalylsulfathiazole in solution is rapidly hydrolyzed to sulfathiazole and phthalic acid. Solutions of sulfathiazole were diazotized simultaneously and used as standards for photoelectric colorimetry. "Total" sulfathiazole was determined by heating the p-toluenesulfonic acid filtrate of blood or urine for 1 hour at 100°C, a procedure adequate for the release of sulfathiazole from the conjugated forms.

The LD₅₀ of succinylsulfathiazole, using the same technique (2), was found to be 5.7 grams per kgm. These data demonstrated that in mice the acute toxicity of succinylsulfathiazole, administered intraperitoneally, was approximately one sixth that of phthalylsulfathiazole. Marshall et al. (6) found that 11 of 15 mice (73%) were killed by the intraperitoneal injection of 0.5 gram per kgm. of sulfanilylguanidine suspended in olive oil.

Intraperitoneal toxicity (sodium salt). The sodium salt of phthalylsulfathiazole in aqueous solution, pH about 7.6 (1.6 to 10 per cent), was injected intraperitoneally (25 cc. per kgm.) into female white mice (Carworth CF₁). The LD₅₀ was approximately 0.8 gram per kgm. of body weight. The signs of toxicity were similar to those seen following the intraperitoneal injection of the acid form suspended in olive oil.

TABLE 1

Mortality and concentration of sulfonamides (in mgm. per 100 cc.) in the blood of mice following the intraperitoneal injection of phthalylsulfathiazole

DOSE mgm./kgm.	NUMBER DEAD/TOTAL	OBSERVED MORTALITY PERCENTAGES	CALCULATED MORTALITY PERCENTAGES	APPROXIMATE TIME OF DEATH hours	MEAN CONCENTRATION IN BLOOD TWO HOURS FOLLOWING INJECTION	
					Free*	Conjugated*
					mgm./100 cc.	mgm./100 cc.
500	0/15	0	0			
625	1/15	7	4	34	6.8	17.8
880	7/15	47	40	36		
1100	12/15	80	83	19	13.5†	33.3†
1375	14/15	93	97	5		
1720	15/15	100	100	4	31.9	89.7

* See footnote, 4.

† These concentrations were actually found in mice given 1000 mgm. per kgm., approximately the LD₅₀.

Chronic toxicity. Repeated parenteral administration. Four monkeys were given daily intraperitoneal injections of the sodium salt of phthalylsulfathiazole, during a period of 10 days. The drug was administered as a 20 per cent aqueous solution (pH 7.6).⁵ One monkey was given 1.0 gram per kgm. of body weight daily, another 0.33 gram per kgm. daily and two animals 0.1 gram per kgm. daily. Determinations of the total erythrocyte counts, the total leucocyte counts, the differential leucocytic counts, the concentration of hemoglobin, plasma proteins and blood urea, the percentage of reticulocytes, and the volume of packed cells were made both before the experiment was begun and at its termination. Determinations of the sulfonamide concentrations in the blood and the amount appearing in the urine were performed at various intervals during the test as tabulated in tables 2 and 3. On the eleventh day the animals were sacrificed and autopsied.

⁵ The aqueous solution of sodium phthalylsulfathiazole (20 per cent) was prepared each day just prior to the injections. The amount of free sulfathiazole present was not in excess of 2 per cent.

TABLE 2

Concentration of sulfonamides (in mgm per 100 cc) in the blood of monkeys following the daily intraperitoneal administration of sodium phthalylsulfathiazole

DAY	HOURS FOLLOWING INJECTION	MONKEY #53 1.0 GRAM PER KG		MONKEY #46 0.33 GRAM PER KG		MONKEY #52 0.1 GRAM PER KG		MONKEY #56 0.1 GRAM PER KG	
		Free*	Conj*	Free	Conj	Free	Conj	Free	Conj
1	½	15.8	11.0			6.3	5.3	2.6	9.1
	1	26.1	21.0			6.9	5.6	2.6	9.3
	1	30.8	21.7			6.7	5.6	1.0	6.7
	2	11.2	31.5			2.0	6.3	0.7	2.7
	4	9.5	28.5			†	0.9	0.5	0.3
	8	3.3	17.1			†		†	
	24	†		†		†		†	
2	½			5.7	22.6				
	1			5.4	20.9				
	1			13.0	10.4			1.9	8.3
	2	12.5	47.6	8.5	9.7	1.7	5.8	0.5	2.2
	4	13.3	31.8	6.3	8.4	†		†	
	24	2.3	6.4	†		†		†	
3	1	18.2	59.0			3.2	11.3	2.3	6.6
	2	14.1	62.4			1.3	6.8	1.2	2.7
	4	12.4	45.2			0.7	0.7	0.8	0.8
	24	5.8	18.7	†		†		†	
4	1	23.2	31.7	13.2	17.6	2.6	0.4	2.1	6.8
	2	37.5	41.1	0.2	13.4	1.9	2.8	0.7	2.0
6	24			†		†			
7	1			6.9	22.7	5.3	11.7	4.4	4.5
	2			3.1	22.5	1.6	5.0	1.1	1.6
	4			2.9	18.4	†		†	
9	24			2.0	3.2	†		†	
10	½	Died		17.1	18.7	5.0	10.0	2.4	10.0
	1			17.0	24.5	5.0	9.5	2.0	0.6
	1			14.4	26.4	3.0	9.9	1.5	7.0
	2			7.6	30.2	3.1	3.2	0.8	2.0
	4			7.0	27.1	0.9	1.2	†	
	8			10.4	18.5	†		†	
	24			2.2	8.6	†		†	

* The values for "free" and "conjugated" drug are expressed as sulfathiazole

† Less than 0.5 mgm per 100 cc

sied, the following tissues were removed for histological examination: liver, gall bladder, kidney, ureter, bladder, spleen, adrenal, thyroid, pancreas, stomach, duodenum, ileum, colon, lung, heart, lymph nodes, sciatic nerve, spinal cord

TABLE 3

Urinary excretion of sulfonamides following the daily intraperitoneal injection of sodium phthalylsulfathiazole for ten days

DOSE	MONKEY AND DAY	HOURS	TOTAL EXCRETION			PERCENTAGE OF ACCUMULATED DOSE EXCRETED
			Free*	Total†	Accumulated total‡	
1.0 gram per kgm. per day (actual daily dose, 8.14 grams)	#53 1st day	0-2½	0.03	1.65	1.65	20
		2½-4	0.02	1.44	2.09	38
		4-8	0.01	0.14	2.23	40
	3rd day	0-1½	0.01	0.18	0.18	2
		1½-4	0.01	0.22	0.40	5
		4-6	0.02	0.42	0.82	10
		6-8	0.01	0.22	1.04	13
		8-24	0.08	1.14	2.18	27
	#46	0-1	0.01	0.29	0.29	14
		1-4	0.03	0.74	1.03	50
		4-8	0.08	0.49	1.52	73
		0-2	0.02	0.20	0.20	10
		2-4	0.01	0.38	0.58	28
		4-8	†	0.15	0.73	35
		8-24	0.03	0.57	1.30	62
		0-2	0.02	0.15	0.15	7
		2-4	†	0.09	0.24	12
		4-8	0.01	0.11	0.35	17
		8-24	0.08	0.72	1.07	51
0.1 gram per kgm. per day (actual daily dose, 0.92 gram)	#52 1st day	0-2	0.01	0.27	0.27	29
		2-4	0.01	0.42	0.69	75
		4-6		0.03	0.72	78
		6-8	†	0.01	0.73	79
	3rd day	0-2	0.01	0.42	0.42	46
		2-4	0.01	0.24	0.66	72
		4-6	†	0.08	0.74	81
		6-8	†	0.01	0.75	82
		8-24	0.01	0.03	0.78	85
	7th day	0-2	0.04	0.28	0.28	30
		2-4	0.01	0.08	0.36	39
		4-8	0.01	0.08	0.44	48
		8-24	0.01	0.02	0.46	50
	10th day	0-2	0.01	0.17	0.17	18
		2-4	0.02	0.32	0.49	53
		4-8	†	0.07	0.56	61
		8-24	0.01	0.02	0.58	63

TABLE 3—(Continued)

DOSE	MONKEY AND DAY	HOURS	TOTAL EXCRETION ^a			PERCENTAGE OF ACCUMULATED DOSE EXCRETED
			Free*	Total†	Accumulated total‡	
			grams	grams	grams	
0.1 gram per kgm per day (actual daily dose 0.45 gram)	#56 1st day	0-2	0.003	0.11		
		2-4	0.001	0.03	0.14	31
		4-24	0.002	0.16	0.30	67
	3rd day	0-2	0.003	0.10		
		2-4	0.01	0.08	0.18	40
		4-8	0.001	0.01	0.10	42
		8-24	0.003	0.01	0.20	44
	7th day	0-2	0.01	0.21	0.21	47
		2-4	0.002	0.06	0.27	60
		4-8	0.002	0.01	0.28	62
		8-24	†	0.02	0.30	67
	10th day	0-2	0.003	0.19	0.10	42
		2-4	0.002	0.06	0.25	46
		4-8	0.002	0.01	0.26	48
		8-24	0.003	0.01	0.27	60

* Expressed as sulfathiazole

† Expressed as phthalylsulfathiazole

‡ Less than 0.01 gram

and bone marrow. All tissues were stained with hematoxylin and eosin, except for the bone marrow which was stained by Maximow's method.

Manifestations of toxicity were noted following the first intraperitoneal injection of 1.0 gram per kgm to a monkey. This animal, a large adult male (8.1 kgm), was extremely difficult to handle. Five minutes after the initial injection vomiting occurred and the animal showed evidence of considerable abdominal discomfort. Approximately three hours after the injection a sample of urine was collected which was slightly reddish in color and from which on cooling there was deposited a large amount of crystals. The injection of the compound on the second day was followed by nausea, borborygmus, eructation, salivation, and vomiting. The animal was unable to sit or stand but lay on the floor of the cage. On succeeding days (the third, 4th, 5th and 6th) no vomiting was observed, but muscular weakness was present. The animal was pale and did not resist handling in the vigorous manner previously noted. The appetite was diminished and a light reddish brown diarrhea was apparent from the fourth day until death occurred during the night of the sixth day.

Table 2 in which are presented the concentrations of free and conjugated sulfonamides found in the blood of these animals shows that considerable absorption of drug took place and that marked accumulation in the body was present by the fourth day. The average concentration in the blood two hours following

administration during a 4 day period was 18.8 mgm. of free and 45.7 mgm. of conjugated sulfathiazole per 100 cc.

In table 3 are presented the urinary concentrations of free and total sulfonamide and the total amount of drug excreted in the urine during 8 hours on the first day and during 24 hours on the third day. These data show that absorption of the sulfonamide from the peritoneal cavity was marked. That this was the case is more adequately demonstrated by the data, presented in the same table, and the excretion of the compound in the urine of monkeys receiving smaller doses. The data on urinary excretion also show that sulfonamide retention occurred, since on the first day 40 per cent of the dose administered was excreted during the succeeding 8 hours, whereas of a similar dose given on the third day only 13 per cent was excreted within the same period of time. On the third day only 27 per cent of the dose administered was excreted during a 24 hour period. The concentration of sulfonamide in the blood on the third day indicated that absorption from the peritoneal cavity was not diminished, in comparison with the absorption on the first day.

Autopsy of the animal described above revealed grossly a small recent infarct in the myocardium, extensive recent bloody exudation in the peritoneal cavity and extensive crystalline deposits in the renal parenchyma associated with crystalline concretions in the renal pelvis. Microscopic examinations bore out the gross findings, and in addition disclosed considerable degenerative changes in the kidneys and liver, and some reduction of the zona glomerulosa and lipid content of the adrenals. The other organs showed no significant lesions.

A second monkey was given daily intraperitoneal injections of 0.33 gram of sodium phthalylsulfathiazole per kgm. of body weight for ten days. This animal survived the test and manifested none of the severe reactions displayed by the monkey which had received the larger dose. There was some loss of appetite during the latter part of the test but this was not as marked as in the previous animal. No nausea or vomiting was observed during the test.

In table 4 have been tabulated the values at the beginning and at the completion of the test for weight, percentage of hemoglobin and plasma proteins, cell volume (hematocrit), blood urea, the total erythrocyte and leucocyte counts and the percentage of reticulocytes. The loss of weight was considered to be associated with the lowered dietary intake rather than with any more specific toxic effect. The decrease in hemoglobin concentration followed the decrease in the erythrocyte count, but this could not be considered significant, since the erythrocyte count was still within the normal range of variation. Further evidence of the lack of significance of the hemoglobin and erythrocytic changes was offered by the normal reticulocytic count and the essentially normal bone marrow. The mean concentration of total sulfonamide in the blood of this animal increased during the course of the experiment (see table 2), whereas the total urinary excretion of sulfonamides decreased during the same period. The percentage of the daily dose which was excreted during the first 8 hours of the second day was 73 per cent (see table 3), 35 per cent during the same period on the seventh day, and 17 per cent within a similar time on the tenth day.

At autopsy the kidneys of this animal were pale and swollen, the collecting tubules were sharply delineated with what appeared to be crystalline deposits of the drug. Except for some nodules in the parenchymia of the lung and six small lipomas in the peritoneal cavity no other gross lesions were found. Microscopically, the kidneys showed degenerative changes in the tubules, glomerular dilatation and hyperplasia of the epithelium of the pelvis. There were minor changes in the liver and parasitic infestation in the lungs; other tissues were essentially normal.

Two monkeys (#52 and #56) given 0.1 gram per kgm. of sodium phthalylsulfathiazole intraperitoneally for ten days, manifested no signs of toxicity during the course of the experiment. Table 4 shows that there were no significant changes in the blood picture of these animals. We consider the total leucocyte

TABLE 4

Changes in weight and in certain blood constituents of three individual monkeys administered sodium phthalylsulfathiazole intraperitoneally daily for 10 days

	0.33 GRAM/KGM /DAY		0.1 GRAM/KGM /DAY		0.1 GRAM/KGM /DAY	
	In t al values	Values on 10th day	In t al values	Values on 10th day	In t al values	Values on 10th day
Weight (kgm.)	6.28	5.6	9.12	8.0	4.5	4.5
Hemoglobin (grams per 100 cc.)	16.0	14.1	15.0	13.0	16.4	14.6
Hematocrit (%)	54.1	44.6	47.8	45.4	46.0	44.5
Plasma protein (grams per 100 cc.)	10.1	8.24	7.42	10.4	8.44	8.81
Blood urea (mgm per 100 cc.)	11.6	22.8	11.6	16.4	20.5	17.6
Erythrocytes (millions per cu mm.)	6.8	5.4	6.0	5.44	6.7	5.32
Leucocytes (thousands per cu mm.)	11.29	15.00	8.44	11.12	6.00	18.20
Reticulocytes (%) (1000 cells counted)	0.8	0.8	0.9	1.3	0.2	0.6

counts usual, since we have found, as has been reported by others (8, 9), that such values in normal monkeys under laboratory conditions are subject to considerable variation. The sulfonamide concentrations attained in the blood of these animals indicated rapid absorption of the drug from the peritoneal cavity. The average concentrations in the blood one hour following the dose were for #52, 4.2 mgm free and 9.5 mgm conjugated and for #56, 2.4 mgm free and 10.5 mgm of conjugated sulfathiazole per 100 cc. In these animals renal excretion remained satisfactory throughout the period of observation. The data presented in table 3 suggest a reduction in the excretory ability of the animals on the seventh day with #52 and on the third day with #56. That these reductions were probably due to decreased fluid intake during those days is evidenced by the increased excretion on the following days of the test when the water intake was known to have been maintained.

At autopsy monkey #52 showed definite evidence of pneumonia, two small cysts on the right kidney and fatty replacement of the bone marrow. Microscopic examination revealed bronchopneumonia with abscess formation, colloid cysts of the kidney, a mild toxic nephrosis, almost complete replacement of the bone marrow with fatty tissue, and some necrosis and calcification of the adrenal medulla and to a lesser degree of the adrenal cortex. All other tissues were normal. The pneumonic process and adrenal changes were probably tuberculous in origin, while the fatty replacement of the bone marrow was a reflection of the age of the animal.

The autopsy of monkey #56 revealed a small ulcer 2 cm. in diameter about 10 cm. distal to the ileocecal junction. The necrotic central area of the ulcer had apparently eroded through the intestinal wall. There were no adhesions nor any evidence of inflammation in the peritoneal cavity. Microscopic examination of sections of tissues from this animal indicated an active bone marrow with moderate fatty replacement; emphysema and anthracosis of the lung; a patch of necrotic mucosa in the colon, which showed an intense cellular reaction, probably attributable to an error in the injection of the compound. In the kidney the same nephrotic changes were seen as in the preceding animal. The toxic nephrosis, which was seen in both animals, was the only anatomical change referable to drug administration. These changes were patchy in distribution, mild in character, and were considered to have been reversible.

Poth (10) had observed no severe manifestations of toxicity in dogs following the intravenous administration of 2 grams of sodium phthalylsulfathiazole per kgm. per day (for 7 days), whereas we found that monkeys exhibit severe toxic signs when the same drug was administered intraperitoneally in the amount of 1 gram per kgm. per day. Preliminary observations have been made on a dog and a monkey given 1-gram per kgm. of sodium phthalylsulfathiazole intravenously every day for 10 days. These preliminary data indicate only that there was a difference in the toxicity of the drug in the two species and that several factors may be involved in this difference.

Chronic Toxicity. Rats. Forty albino rats (Wistar strain), each of approximately 100 grams in weight, were divided into groups of 10 each in such a manner that the average weight for each group was similar. One group was fed a powdered commercial ration⁶; a second group, the same ration with the addition of 2 per cent phthalylsulfathiazole; a third group, the same with 5 per cent phthalylsulfathiazole; and the fourth group, the same with 10 per cent phthalylsulfathiazole. All animals were kept in individual cages and were allowed free access to the diet for 30 days. The animals were weighed once weekly; drug consumption and the concentration of sulfonamides in the blood were measured on the seventh and twenty-first days of the experiment. At the completion of the experiment (31 days) all rats were autopsied and various tissues (kidney, liver, spleen, heart, stomach, duodenum, ileum, cecum, colon, adrenal, lung, pancreas, thyroid and bone marrow) from each of five rats of each group were examined histologically.

⁶ Purina Dog Chow Checkers.

Reference to table 5 shows that on a ration containing 10 per cent phthalylsulfathiazole the growth rate of the rats was depressed. However, there were no other evidences of toxicity; all animals survived the experiment and histological examination of the tissues from these animals failed to disclose any evidence of pathological change.

The growth of animals given rations containing 2 per cent and 5 per cent of phthalylsulfathiazole appeared to be greater than that of the control rats. Measurements of the 24 hour food intake of the animals indicated that the drug-fed animals ate slightly more than the control group. The average 24 hour food intakes were, for the control group, 15.8 grams; the 2 per cent drug group, 18.0 grams; the 5 per cent drug group, 18.8 grams; and the 10 per cent drug group, 17.2 grams. (These figures are corrected for drug content.) There was evidence of an intercurrent, low grade infection (paratyphoid) in the control group.

TABLE 5

The effect of phthalylsulfathiazole on the weight gain of white rats

DIET AND SUPPLEMENT	INITIAL WEIGHT	INCREASE IN WEIGHT
	grams	grams
Basal (commercial ration)	96 \pm 3.6 (10 animals)	95 \pm 12.1 (8 animals)
2% Phthalylsulfathiazole*	96 \pm 4.1 (10 animals)	121 \pm 10.5 (7 animals)
5% Phthalylsulfathiazole*	96 \pm 4.0 (10 animals)	113 \pm 8.1 (8 animals)
10% Phthalylsulfathiazole*	96 \pm 4.4 (10 animals)	76 \pm 4.5 (10 animals)

* The drug, in the amounts indicated, was incorporated in the powdered commercial ration, Purina chow.

Diarrhea was noted in several of these rats and lesions suggestive of paratyphoid were seen in the liver and in the colon of one. Three of the animals in the 2% group died during the test, two on the 7th day and one on the 10th day. One of these animals was injured during handling, the others showed progressive weight loss and bloody diarrhea prior to death. In the 5% group one rat died on the 13th day and one died on the 21st day, both animals developed diarrhea and lost weight as the test progressed. Microscopic examinations were not made on any of the rats which died during the test period.

None of the other animals in the drug-fed groups (2 per cent and 5 per cent) showed any evidences of toxicity during the experiment. Microscopic examination of tissues taken at autopsy revealed no significant lesions in any of the tissues.

The average food consumption and blood sulfonamide concentrations in these rats are presented in table 6.

It will be noted that on the 7th day the rats on the 10 per cent drug diet received an average of 1.7 grams of drug per day, which represented at the average weight of the animals, approximately 17 grams per kgm. per day of phthalylsulfathiazole. On the 21st day the average daily intake for this same group represented approximately 12 grams per kgm. per day. The highest individual concentration in the blood attained in this group was 3.6 mgm. of free and 3.1 mgm. of conjugated sulfathiazole per 100 cc.

Chronic Toxicity. Monkeys—Technique of experiment. Eight monkeys (*Macaca mulatta*) were stomach-tubed at four hour intervals daily for 30 days. Six of these animals were given phthalylsulfathiazole in varying doses freshly suspended in 0.5 per cent mucilage of tragacanth. The dosage regime (six doses daily) was as follows: 2 monkeys received 0.5 gram/kgm. per day; 2 monkeys received 1.5 grams/kgm. per day and 2 monkeys received 5.0 grams/kgm. per day; similarly, 2 monkeys served as controls, each of which was concurrently

TABLE 6

Average food and drug consumption and mean blood concentrations of sulfonamides attained in rats receiving phthalylsulfathiazole in the diet

DIET	SEVENTH DAY				TWENTY-FIRST DAY			
	Food consumed	Drug consumed	Mean blood concentration		Food consumed	Drug consumed	Mean blood concentration	
			Free*	Conjug.*			Free*	Conjug.*
	gm.	gm.	mgm./100 cc.		gm.	gm.	mgm./100 cc.	
2% Phthalylsulfathiazole	17.6	0.35	1.2	0.5	19.6	0.39	1.3	1.1
5% Phthalylsulfathiazole	16.7	0.84	1.9	1.2	20.0	1.0	2.0	2.5
10% Phthalylsulfathiazole	16.6	1.7	3.1	3.1	19.1	1.9	3.3	3.5

* Expressed as sulfathiazole.

intubated with 20 cc. each of 0.5 per cent tragacanth. Twice daily the animals were fed on oranges and Purina Fox Chow; water was given *ad libitum*. In addition each monkey was given three cc. of a vitamin concentrate⁷ once daily throughout the test. Determinations of the total erythrocyte counts, the total leucocyte counts, the reticulocyte count, the differential leucocyte counts, the percentage of haemoglobin and of plasma protein, the packed cell volume (hematocrit), the prothrombin time and the concentration of free and total sulfonamides in the blood were made at the beginning of the experiment and at intervals during its course. The concentration of the drug in the urine was determined at such intervals as it was possible to obtain uncontaminated urine specimens. In certain instances, catheterization was resorted to in order to obtain such samples. The animals (excluding one of the controls) were sacrificed on the 31st day of the experiment by light anaesthesia with a barbiturate and subse-

⁷ The approximate analysis of the vitamin concentrate (3 cc.) was as follows: thiamin hydrochloride, 1.8 mgm.; riboflavin, 0.6 mgm.; nicotinic acid, 0.7 mgm.; pyridoxine hydrochloride, 0.3 mgm.; pantothenic acid, 1.3 mgm.

quent exsanguination. Subsequent to observations for gross lesions the following tissues were taken for histopathological examination: liver, gall bladder, kidney, ureter, bladder, spleen, adrenal, thyroid, pancreas, stomach, duodenum, ileum, colon, lung, heart, lymph nodes, sciatic nerve, spinal cord, and bone marrow. These tissues were stained with haematoxylin and eosin; in addition, the bone marrow was stained by Maximow's method, the spinal cord and nerves were sectioned by the freezing technique and examined under polarized light for myelin changes.

During the test careful observations were made as to the general state of health and behavior of the animals; particular attention was given to nausea, vomiting, appetite, weight loss and activity. Regurgitation of a dose occurred in two instances following gastric intubation but was not associated with any evidence of nausea. Diarrhea was not present in any of the monkeys. The feces of the monkeys fed phthalylsulfathiazole had less odor than normal, the feces of the monkeys on the two highest doses being particularly free of odor and colored white with the drug.

Weight loss was noted in one of the animals receiving 1.5 grams per kgm. of phthalylsulfathiazole daily, in both animals receiving 5.0 grams per kgm. of phthalylsulfathiazole daily, and in one of the control animals. These animals were the heaviest at the beginning of the experiment and most of the weight loss occurred in the first two weeks of the experiment. It seems probable that the constant handling of these animals, 6 times daily during the thirty day period, was responsible for some of the weight loss. However, the two monkeys on 5.0 grams/kgm. per day lost 24 per cent and 23 per cent respectively of their initial weights. It was noted that both of these animals showed loss of appetite; in one of the two animals this was associated with general weakness. A third drug-treated animal lost only 6 per cent of the original weight; this was considered to have no significance since one of the controls lost 10 per cent of its original weight.

On the ninth day of the test all the experimental and control animals showed reductions in the total number of erythrocytes. The greatest reductions were seen in one of the controls and in one of the monkeys given 1.5 grams per kgm.; in each case the reduction was to 68 per cent of the original count. In these two animals significant increases in the percentage of reticulocytes occurred (5.4 per cent). There was presumptive evidence that this was a nutritional anemia in the control animal; its occurrence in the animal given 1.5 grams per kgm. of phthalylsulfathiazole daily was probably related to the presence of persistent bleeding from a damaged tooth socket. This bleeding was aggravated each time the animal was caught for intubation and was not controlled until two weeks had passed. We have noticed a general trend toward a reduction of the erythrocyte count of all monkeys, experimental and control, during the first week of previous experiments of this nature. This fact and the general return of the counts toward normal levels during the remainder of the experiment indicate the non-specific nature of the phenomenon.

The hemoglobin concentrations showed no significant changes at the completion of the test. Three animals showed some reduction in hemoglobin concen-

trations at the end of the first week. One of these animals had a persistently bleeding tooth socket, as was discussed previously. The other animals had slight reductions in the erythrocyte counts concurrent with the decreased hemoglobin concentrations. These reductions were not considered to have been due to the drug treatment.

Hematocrit values remained within normal limits throughout the experiment. The plasma protein concentrations were somewhat reduced from their initial values at the termination of the experiment but since the reduction occurred to an even greater extent in the control animals these changes cannot be attributed to the drug treatment given. Total leucocyte counts and differential leucocyte counts were followed throughout the experiment; no deviations attributable to the drug were observed. Since the total leucocyte count in normal monkeys under the conditions of an experiment such as this show remarkably wide variations, little reliance should be placed on such figures.

TABLE 7

Average sulfonamide concentrations (in mgm. per 100 cc.) in the blood of monkeys given phthalylsulfathiazole orally, in six divided doses, daily for thirty days

DOSE*	2 HOURS		4 HOURS	
	Free†	Total†	Free†	Total†
	mg.			
0.5	0.59	0.83	‡	0.62
0.5	‡	0.67	‡	0.58
1.5	0.55	1.13	‡	0.80
1.5	‡	0.82	‡	0.71
5.0	1.03	1.46	0.83	1.43
5.0	0.83	1.39	0.52	1.20

* Doses are expressed in grams per kilogram of body weight per day.

† Expressed as sulfathiazole.

‡ Trace, less than 0.5 mgm. per 100 cc.

The concentrations of free and total sulfonamides in the blood stream of monkeys receiving phthalylsulfathiazole remained low throughout the experiment. The maximum blood concentrations attained two hours following intubation in the monkeys receiving 5.0 grams per kgm. per day were 1.38 mgm. of free and 1.79 mgm. of total sulfathiazole per 100 cc. Slightly lower concentrations in the blood were always obtained at 4 hours than at 2 hours following intubation (see table 7). The low blood levels indicate that phthalylsulfathiazole was either absorbed very poorly from the gastrointestinal tract or was excreted by the kidneys with great rapidity. As was found with succinylsulfathiazole, phthalylsulfathiazole was both poorly absorbed and rapidly excreted.

A wide variation in the percentage of the ingested dose excreted in the urine during a 24 hour period occurred (table 8). For example, one of the animals which received 1.5 gram per kgm. daily excreted 3.6 per cent of the ingested dose, while a second monkey on a similar dose excreted only 0.8 per cent. Great

difficulty was experienced in obtaining suitable data of the type described in table 8 because of the difficulty in securing urine uncontaminated with feces containing very large amounts of unabsorbed sulfonamide. The data presented, however, show that phthalylsulfathiazole must have been very poorly absorbed from the gastrointestinal tract of the monkey since the sulfonamide content of the urine, as well as that of the blood was consistently low.

On the thirty first day of the test the animals were autopsied. None of the lesions which were found could be accounted for by the treatment to which the animals were exposed. The lesions found, and which will be described in the following section, might be expected in any group of monkeys of varying ages and antecedents.

Pathological Findings The data in this section are presented in summary form, in the following order: Number of monkey, sex, dose in grams per kilo gram per day (six divided doses given every 4 hours), condition of the animal.

TABLE 8

Sulfonamides in the urine of monkeys following the oral administration of phthalylsulfathiazole

DOSE	ACTUAL DAILY DOSE INGESTED	AVERAGE URINARY CONCENTRATION OF DRUG		ESTIMATED DAILY URINARY OUTPUT	TOTAL* DAILY EXCRETION OF DRUG†	PERCENTAGE OF* INGESTED DOSE EXCRETED IN A 24 HOUR PERIOD
		Free†	Total†			
grams/kgm./day	grams	mgm./100 cc		cc	grams	
0.5	1.74	12.3	34.0	300	0.102	5.9
0.5	2.25	12.4	33.5	600	0.201	8.9
1.5	8.7	43.7	125.0	250	0.313	3.6
1.5	7.7	7.0	21.0	300	0.063	0.8
5.0	30.0	32.6	81.0	300	0.243	0.8
5.0	29.0	21.2	65.0	300	0.195	0.7

* The total daily excretion and the percentage of ingested dose excreted daily are calculated from the estimated daily volume of urine.

† Expressed as sulfathiazole.

‡ Expressed as phthalylsulfathiazole.

during the test, weight change, number of erythrocytes (rbc) in millions per cmm, number of leucocytes (wb) in thousands per cmm, the concentration of hemoglobin (hb) in grams per 100 cc, reticulocytes (ret) in per cent of 1000 cells, the packed cell volume (hmet) in per cent, percentage prothrombin (pt), the concentration of plasma proteins (pp) in grams per 100 cc, gross and microscopic findings at autopsy.

Monkey #40 Female, control, 20 cc of 0.5 per cent tragacanth every four hours, survived and well, 4.1 kgm increased to 4.7 kgm, rbc, 6.76 to 5.13, wb, 13.44 to 11.68, hb, 14.1 grams to 15.1 grams, ret, 0.4% to 0.7%, hmet, 45.1% to 56.0%, pt, 106% to 97%, pp, 10.1 grams to 7.4 grams, no gross lesions microscopic all tissues normal.

Monkey #48 Female, 0.5 gram, survived and well, 3.46 kgm to 3.85 kgm, rbc, 6.1 to 5.6, wb, 11.72 to 9.58, hb, 14.0 grams to 14.8 grams, ret, 1.0% to 0.4%, hmet, 45.8% to 52.0%, pt, 25.0% to 24.5%, pp, 9.1 grams to 8.4

grams; no gross lesions; microscopic: lung—slight emphysema; heart muscle stains irregularly; other tissues normal.

Monkey #47. Female; 0.5 gram; survived and well; 4.5 kgm. to 4.94 kgm.; r.b.c., 5.9 to 5.51; w.b.c., 15.28 to 14.82; hb, 13.2 grams to 12.5 grams; ret., 0.6% to 0.7%; hmet., 43.3% to 47.0%; pt., 85.0% to 80.0%; pp., 10.5 grams to 7.8 grams; no significant gross lesions; microscopic: spleen—some dilatation of sinusoids; other tissues normal.

Monkey #49. Female; 1.5 grams; survived and well; 5.8 kgm. to 5.45 kgm.; r.b.c., 6.76 to 5.13; w.b.c., 13.4 to 11.68; hb, 14.2 grams to 13.2 grams; ret., 0.4% to 0.7%; hmet., 46.4% to 50.0%; pt., 96% to 102%; pp., 9.1 grams to 7.8 grams; no gross lesions; microscopic: thyroid—small colloid adenoma; spleen—hyperplasia; other tissues normal.

Monkey #41. Female; 1.5 grams; survived and well; 5.12 kgm. to 5.53 kgm.; r.b.c., 5.72 to 5.57; w.b.c., 13.32 to 13.4; hb, 13.1 grams to 13.4 grams; ret., 0.6% to 0.5%; hmet., 42.0% to 50.0%; pt., 76% to 120%; pp., 9.5 grams to 8.5 grams; no gross lesions; microscopic: heart—diffuse small round cell infiltration throughout myocardium; spleen—some hyperplasia; other tissues normal.

Monkey #50. Female; 5.0 grams; survived, but showed appetite loss during last two weeks of test and general weakness during last week of test; 5.96 kgm. to 4.54 kgm.; r.b.c., 6.12 to 5.39; w.b.c., 15.42 to 12.36; hb, 13.9 grams to 14.6 grams; ret., 0.6% to 0.5%; hmet., 47.7% to 50.0%; pt., 106% to 104%; pp., 8.1 grams to 7.1 grams; gross lesions: bone marrow—marked fatty replacement; liver—pale and mottled, increased fat content; stomach—four small areas (2 to 3 cm. in diameter) in the fundus which showed increased vascularity; microscopic: liver—fatty replacement of parenchymatous cells, liver cords separated; kidney—glomeruli relatively bloodless and contracted, tubules somewhat dilated and occasionally containing erythrocytes; lung—few small foci of round cell infiltration, small amount of edema; bone marrow—moderate adipose replacement; other tissues normal.

Monkey #51. Female; 5.0 grams; survived, but showed some loss of appetite during last week of test; 5.8 kgm. to 4.5 kgm.; r.b.c., 6.26 to 5.87; w.b.c., 6.4 to 8.4; hb, 13.0 grams to 13.7 grams; ret., 0.8% to 0.9%; hmet., 42.5% to 47.0%; pt., 108% to 140%; pp., 9.5 grams to 7.6 grams; no gross lesions; microscopic: jejunum—hypersecretion of mucus; stomach—some hyperplasia of mucosa; other tissues normal.

Monkey #46. Female; control; 20 cc. of 0.5% tragacanth every 4 hours; survived and well; 7.2 kgm. to 6.5 kgm.; r.b.c., 5.82 to 5.63; w.b.c., 10.08 to 10.56; hb, 15.0 grams to 16.1 grams; ret., 0.5% to 0.5%; hmet., 46.5% to 56%; pt., 104% to 105%; pp., 9.5 grams to 7.7 grams. This animal was not sacrificed.

SUMMARY

Studies of the acute and chronic toxicity of a new sulfathiazole derivative, 2-(N⁴-phthalyl-sulfanilamido)-thiazole may be summarized as follows.

1. No evidences of toxicity were observed in white mice following the oral administration of suspensions of phthalylsulfathiazole (10 grams per kgm.).

Two hours following gastric intubation the average concentrations in the blood produced by this dosage were 2.4 mgm of free and 4.1 mgm of total sulfathiazole per 100 cc

2 The LD_{50} following the intraperitoneal administration in white mice of phthalylsulfathiazole suspended in olive oil was approximately 0.9 gram per kgm. Following the intraperitoneal injection, in olive oil, of 1.0 gram per kgm of phthalylsulfathiazole, the concentrations in the blood averaged 13.5 mgm of free and 46.8 mgm of total sulfathiazole per 100 cc. The intraperitoneal administration into white mice of aqueous solutions of sodium phthalylsulfathiazole gave an LD_{50} of 0.8 gram per kgm.

3 Intraperitoneal injections into monkeys of aqueous solutions of the sodium salt of phthalylsulfathiazole disclosed that a dose of 0.1 gram per kgm per day for 10 days caused no toxic manifestations with the exception of mild toxic nephrosis and gave rise to average 1 hour concentrations in the blood of 4.2 mgm of free and 13.7 mgm of total sulfathiazole per 100 cc. An intraperitoneal dose of 0.33 gram per kgm per day for 10 days was followed by some retention of sulfonamides and tissue damage, a dose of 1.0 gram per kgm per day resulted in marked toxic manifestations, sulfonamide retention, severe kidney damage and death on the sixth day.

4 Rats showed no depression of the growth rate or any other manifestations of toxicity when fed for 30 days on a commercial ration to which phthalylsulfathiazole was added to the extent of 2 or 5 per cent, at a 10 per cent level phthalylsulfathiazole caused a depression of the growth rate but no other toxic manifestations.

5 Six monkeys given phthalylsulfathiazole orally, every four hours for 30 days, in doses up to 5.0 grams per kgm per day, survived the test period and showed no significant changes in the normal values of the various blood constituents and no histopathological changes attributable to the drug. In the two monkeys which received the highest dose (5.0 g./kgm./day) only anorexia and weight loss were noted. The average 4 hour blood concentration in the monkeys receiving the maximum dose was 0.7 mgm of free and 1.3 mgm of total sulfathiazole per 100 cc. The average proportion of the ingested dose which was excreted in the urine during each 24 hour period was estimated to be 3.5 per cent for the group with a minimum of 0.7 per cent and a maximum of 9 per cent.

The evidence presented permits the conclusion that the absence of toxic manifestations following the oral administration of phthalylsulfathiazole is attributable to the very low concentration produced in the blood and other tissues. These low blood levels result from the retention in the gastro intestinal tract of all but small amounts of the drug and the rapid excretion by the kidneys of that which is absorbed.

BIBLIOGRAPHY

- (1) POTR, E. J., KNOTTS, F. L., LEE, J. T., AND INUI, F. *Arch. Surg.*, 44, 187, 1942.
- (2) a. WELCH, A. D., MATTIS, P. A., AND LATVEN, A. R., *This Journal* 75, 231, 1942.
b. POTR, E. J., AND KNOTTS, F. L., *Proc. Soc. Biol. and Med.* 48, 129, 1941.

- (3) POTH, E. J., AND ROSS, C. A., Fed. Proc., 2: 89, 1943.
- (4) POTH, E. J., Texas State J. Med., 39: 369, 1943.
- (5) BRATTON, A. C., AND MARSHALL, E. K., JR., J. Biol. Chem., 128: 537, 1939.
- (6) MARSHALL, E. K., JR., BRATTON, A. C., WHITE, H. J., AND LITCHFIELD, J. T., JR., Bull. Johns Hopkins Hospital, 67: 163, 1940.
- (7) BEHRENS, B., ARCH. EXPTL. PATH. PHARMAC., 140: 237, 1929.
- (8) SHUKERS, C. F., LANGSTON, W. C., AND DAY, P. L., Folia haematol., 60: 416, 1938.
- (9) TALIAFERRO, W. H., AND KLÜVER, C., J. Infect. Dis., 67: 121, 1940.
- (10) POTH, E. J., Texas Report on Biol. and Med., Texas Rep. Biol. and Med., 1: 345, 1943.

INHIBITORY EFFECT OF SULFONAMIDES ON THE ACTION OF NICOTINE IN THE ISOLATED INTESTINE

E P PICK¹, G W BROOKS AND H UNNA

From the Laboratories of the Mount Sinai Hospital, New York N Y and the Merck Institute for Therapeutic Research, Rahway N J

Received for publication February 7, 1944

Extensive investigations on the action of sulfonamides in the living organism and in vitro have produced a wealth of information about the metabolic fate of these compounds in the host and their mode of action on bacteria. However, aside from numerous studies on the absorption, tissue concentration and excretion of the sulfonamides and reports on their effect on the hematopoietic system, there are few data on the effect of these substances on the organism. In view of the absence of any striking pharmacologic effects upon circulation, respiration or the smooth muscle of the host, sulfanilamide and its derivatives are regarded as rather inert substances, at least in concentrations which are therapeutically effective. Acute and chronic overdosage of these compounds affects mainly the nervous system. Rabbits, cats and dogs poisoned with sulfanilamide exhibited muscular weakness, ataxia, athetotic movements, rigidity and convulsions before they became comatose. On autopsy degeneration in some neurones of the spinal cord and in certain cells of the cortex and mid brain were found (1, 2). Similar toxic reactions of cerebral origin have also been reported in man. Furthermore, local application of certain sulfonamides to cranial injuries may produce generalized convulsions in man (3) and dogs (4) and striking changes in the electrical records of the brain of monkeys (5) and cats (6). Peripheral nerves may also be affected by sulfonamides as evidenced by numerous reports on neuritis following the use of sulfanilic acid, sulfanilamide in man.

Apart from these toxic effects, various sulfonamides in otherwise non toxic doses have been found to influence the action of other drugs. Thus, the administration of sulfapyridine and other sulfonamides potentiates markedly the effect of papaverine (7), codeine (8), morphine (8), and barbiturates (9, 10). Moreover, the brain of mice which received sulfapyridine showed an increase in permeability for methylene blue, a finding suggesting an explanation for the increased susceptibility of sulfonamide treated animals to alkaloids and narcotics (11).

In view of these reports on the effect of sulfonamides on the central nervous system, a study of their action upon autonomous nerves seemed to us of interest. In the following we wish to report experiments on the isolated intestine which were mainly concerned with the effect of sulfonamides upon the action of nicotine.

METHODS Rabbits and guinea pigs were killed by a blow on the neck. Pieces of the jejunum measuring about 3 cm. in length were suspended according to Magnus' method in a

¹ Fellow of the Dazian Foundation for Medical Research

modified Ringer solution² in cylinders of 100 cc. capacity. The cylinders were immersed in a temperature bath maintained at $38 \pm 0.5^\circ\text{C}$, and the nutrient solution was continuously aerated with oxygen. Aqueous solutions of sulfanilamide and of the sodium salts of sulfathiazole, sulfamerazine and sulfadiazine were used. The Ringer solution had a pH of 7.5. The addition of the sulfonamides increased the pH to between 8 and 8.5 depending on the amounts added. Concentrations of 100 mgm. per 100 cc. of the different compounds had the following pH: sulfanilamide 7.9; sodium sulfathiazole 8.4; sodium sulfamerazine 8.1 and sodium sulfadiazine 8.1.

Nicotine was used as an aqueous solution of nicotine base. Usually, concentrations from 0.25 to 1 mgm. per 100 cc. produced a marked contraction of the rabbits intestine which, however, was not sustained. The guinea pig's intestine was more sensitive to nicotine than that of rabbits; a marked increase in tone was obtained by the addition of 0.05 to 0.25 mgm. per 100 cc. of nicotine. These concentrations had little effect upon the frequency of the contractions of the intestine. The sensitivity of the intestine for nicotine was determined in each preparation before the addition of other drugs and repeatedly throughout the test in order to guard against changes in the sensitivity of the preparation. After each test, the nutrient solution was exchanged at least twice. The reaction to nicotine remained unaltered when the pH of the Ringer solution was increased to 8 and 8.5 respectively by the addition of 0.08 to 0.15 cc. of a 1% solution of sodium hydroxide per 100 cc. The intestine recovered quickly from the effects of nicotine and of the other compounds tested; once or twice repeated washing with Ringer solution restored its original motility. Other drugs tested in conjunction with the sulfonamides were adrenalin, acetylcholine, lentin, prostigmine, histamine, barium chloride and para-aminobenzoic acid.

RESULTS. *Antagonistic effect of sulfonamides on nicotine.* The addition of any of the sulfonamides employed in this study decreased markedly the reaction of the intestine to a subsequent administration of nicotine. Lower concentrations of the sulfonamides decreased the amplitude of the nicotine contraction whereas higher concentrations completely prevented the effect of nicotine. The effect of the sulfonamides was reversible; removal of the drug by replacement with fresh Ringer solution restored the sensitivity of the intestine to nicotine. The various sulfonamides, however, differed quantitatively in their effectiveness to antagonize nicotine. Sodium sulfathiazole and sodium sulfamerazine sometimes caused a definite decrease of the nicotine effect in concentrations of 12.5 mgm. per 100 cc.; complete suppression of the nicotine effect was obtained with 25 to 100 mgm. per 100 cc. (figs. 1, 2, 3). Sulfanilamide was almost equally effective, whereas sodium sulfadiazine was significantly less effective than any of the other compounds (table 1).

Failure of p-aminobenzoic acid to counteract the effect of sulfonamides. The foregoing observations suggested the question whether compounds which are known to antagonize the bacteriostatic effect of the sulfonamides are capable of reversing the effect of the sulfonamides on nicotine. For this purpose p-aminobenzoic acid was added to the Ringer solution either before or after the administration of sodium sulfathiazole. In neither case did p-aminobenzoic acid (10-100 mgm. per 100 cc., influence the effectiveness of sodium sulfathiazole in inhibiting the nicotine action.

Effect of sulfonamides on the action of other drugs. The influence of the sodium

² The Ringer solution used had the following composition per cent: NaCl 0.9; KCl 0.042; CaCl₂ 0.024; MgCl₂ 0.005; NaHCO₃ 0.05; and dextrose 0.05.

sulfathiazole on the action of drugs other than nicotine was studied on the isolated intestine of both guinea pigs and rabbits in numerous tests with adrenalin, acetylcholine, lentin (erythaminoyl cholme), prostigmine and histamine. Regardless of the presence of sulfonamides in amounts suppressing the nicotine effect completely, the effects obtained with adrenalin (0.1 mgm per 100 cc), acetylcholine (10-20 micrograms per 100 cc), lentin (10-25 micrograms per 100 cc) (fig. 4) and prostigmine (0.1 mgm per 100 cc) remained unchanged. The action of bromine chloride (5-10 mgm per 100 cc) and of histamine (40-100 micrograms per 100 cc) was likewise unaltered by the administration of sodium sulfathiazole (fig. 5).

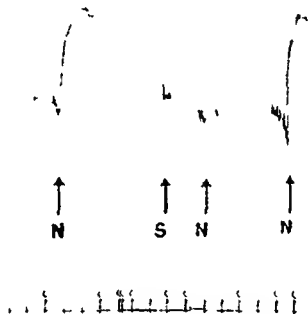


FIG. 1. EFFECT OF SODIUM SULFATHIAZOLE ON THE ACTION OF NICOTINE

Time intervals—1 min
100 cc

Failure of sulfanilamides to influence the action of nicotine on striated muscle
The possibility remained that sulfonamides might inactivate nicotine in vitro. In order to investigate this question, the efficacy of mixtures of sodium sulfathiazole and nicotine incubated for 2 hours at 37°C was tested on frogs. The characteristic attitude of frogs poisoned with nicotine (rigidity and crossing of forelegs, abduction of the thighs and flexion of the hind legs) was obtained with doses of 8-40 mgm nicotine per kgm injected intraperitoneally, and the same effects were caused by the nicotine solution incubated with sulfathiazole. The effective dose range of nicotine was not altered by the addition of sulfathiazole. The results, therefore, excluded the possibility of an interaction between sulfathiazole and nicotine in vitro. They further indicated that simultaneous injections of sulfathiazole did not inhibit the action of nicotine upon skeletal muscle.

Another series of experiments was carried out on the isolated abdominal muscle of the frog suspended in Ringer³ solution. Sulfathiazole and nicotine were tested in doses comparable to those employed on the isolated intestine. The administration of sodium sulfathiazole (in doses up to 100 mgm. per 100 cc.),

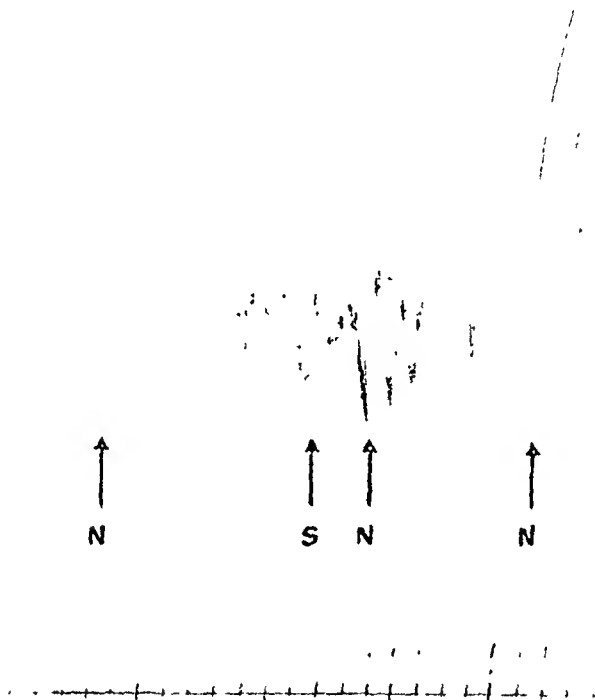


FIG. 2. EFFECT OF SODIUM SULFATHIAZOLE ON THE ACTION OF NICOTINE

Isolated intestine of the rabbit.

N—nicotine 0.5 mgm. per 100 cc.

S—sodium sulfathiazole 25 mgm. per 100 cc.

Time intervals—1 min.

however, did not influence the action of nicotine upon the abdominal muscle (fig. 6).

Discussion. The antagonistic effect of sulfonamides upon the nicotine action in the isolated intestine is not counteracted by para-aminobenzoic acid which negates the bacteriostatic effect of sulfonamides. In this respect our observa-

³ The Ringer solution used had the following composition per cent: NaCl 0.65, KCl 0.02; CaCl₂ 0.02, NaHCO₃ 0.01.

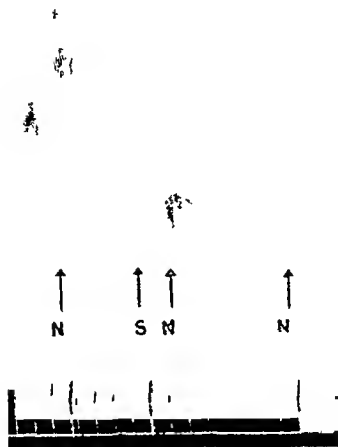


FIG. 3 EFFECT OF SODIUM SULFAMERAZINE ON THE ACTION OF NICOTINE

Isolated intestine of the rabbit

N—nicotine 0.5 mgm. per 100 cc

S—sodium sulfamerazine 50 mgm. per 100 cc

Time intervals—1 min

TABLE I

Concentrations of sulfonamides preventing the effect of nicotine on the isolated intestine of the rabbit

	NO. OF EXPERIMENTS	EFFECTIVE CONCENTRATION (MGM. PER 100 CC.)	
		Mean	Range
Sodium sulfathiazole	63	50	25-100
Sodium sulfamerazine	15	50	25-100
Sulfanilamide	5	60	40-80
Sodium sulfadiazine	9	150*	

*(partial inhibition only)

tions are similar to the finding that the changes in thyroid glands caused by certain sulfonamides are not influenced by para aminobenzoic acid (12). Thus, they present a further indication that effects of sulfonamides other than in bacterial growth are not neutralized by the administration of para aminobenzoic acid.

The observation that sulfathiazole fails to inhibit the action of nicotine on the abdominal muscle of the frog indicates that the action of nicotine on the receptive substance of the striated muscle may not be analogous to that on ganglia and the smooth muscle. Experiments on cats to be published later demonstrated that the action of nicotine upon the vasomotor and respiratory centers is also not influenced by sulfonamides. Furthermore, nicotine (0.1%) failed to inhibit the bacteriostatic effect of sulfathiazole on *E. coli* in vitro.⁴

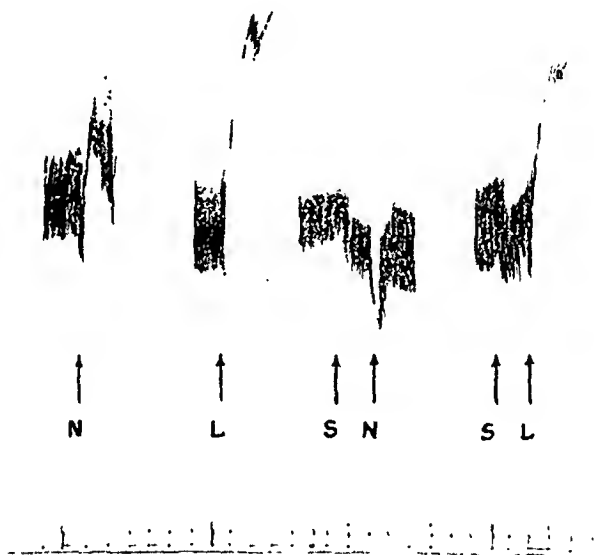


FIG. 4. EFFECT OF SODIUM SULFATHIAZOLE ON THE ACTION OF NICOTINE AND LENTIN

Isolated intestine of the rabbit.

N—nicotine 0.5 mgm. per 100 cc.

S—sodium sulfathiazole 100 mgm. per 100 cc.

L—lentin 0.01 mgm. per 100 cc.

The action of sulfonamides which inhibits the effect of nicotine on para-sympathetic ganglia does not affect the function of the nerve endings of the intestine nor that of the smooth muscle itself, since drugs stimulating sympathetic or para sympathetic nerve endings remain effective and other drugs presumably acting directly upon the smooth muscle cell likewise elicit normal responses in the presence of sulfonamides.

In accordance with Langley's investigations it is generally assumed that nicotine exerts its action upon the intestine through the mediation of the ganglia

⁴ We are indebted to Dr. H. J. Robinson for the bacteriological test.

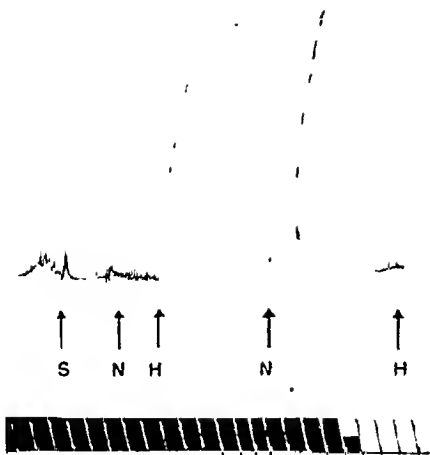


FIG 5 EFFECT OF SODIUM SULFATHIAZOLE ON THE ACTION OF NICOTINE AND HISTAMINE

100 cc

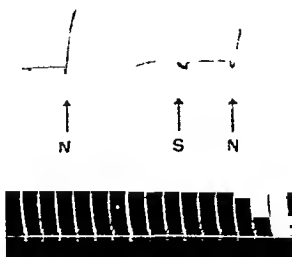


FIG 6 FAILURE OF SODIUM SULFATHIAZOLE TO INHIBIT THE ACTION OF NICOTINE UPON STRIATED MUSCLE

Isolated abdominal muscle of the frog

N—nicotine 0.5 mgm. per 100 cc

S—sodium sulfathiazole 100 mgm. per 100 cc

of the plexus of Auerbach. It appears, therefore, possible that the inhibition of its action by sulfonamides is caused by a blocking of the function of these ganglia, particularly so since an influence of the sulfonamides upon the peripheral end organs could not be established. The inhibition of the nicotine action in the isolated intestine by sulfonamides may represent an analogy to effects of the sulfonamides upon certain enzyme systems, which have already been established with regard to their bacteriostatic action. The bacteriostatic effect of sulfonamides appears to be based upon the inhibition of enzymatic processes which are indispensable for the metabolism of certain microorganisms. Their goiterogenic effect appears to be caused by the inhibition of the synthesis of thyroxine (13, 14). It has also been reported that sulfanilamides inhibit the action of carbonic anhydrase (15) and reduce the inorganic catalytic actions, effects which are not counteracted by para-aminobenzoic acid nor nicotinamide (16). In view of these observations which point to a primary effect of sulfonamides upon enzyme systems the assumption may be entertained that the effect of sulfonamides upon the action of nicotine likewise is caused by an inhibitory effect upon catalytic processes which may be essential in the function of the ganglia and in the action of nicotine upon these ganglia. If the nicotine action were localized not in the ganglia but in the smooth muscle itself, the assumption would be that its action on the receptive or responsive mechanism of the muscle is of a complex nature depending on a catalyst which can be blocked by sulfonamides.

SUMMARY

1. Sulfonamides inhibit the effect of nicotine on the isolated intestine of rabbits and guinea pigs.
2. Sodium sulfathiazole, sodium sulfamerazine and sulfanilamide are more effective in inhibiting the action of nicotine than sodium sulfadiazine.
3. Para aminobenzoic acid fails to antagonize the effect of sulfonamides upon the action of nicotine.
4. The effect of adrenalin, acetylcholine, lentin, prostigmine, histamine, and barium chloride on the isolated intestine is not influenced by sulfonamides.
5. Sulfathiazole does not influence the toxic manifestations of nicotine in frogs, nor does it inhibit the action of nicotine upon the striated muscle of the frog.

The valuable technical assistance given by Grace R. Peters is appreciated.

REFERENCES

- (1) HAWKING, *Lancet*, **2**, 1019, 1937.
- (2) CUSTER, FORSTER, LAMOTTE, PATTON AND PHINNEY, *Arch. Path.*, **26**, 904, 1938.
- (3) WATT AND ALEXANDER, *Lancet*, **1**, 493, 1942.
- (4) PILCHER, ANGELUCCI AND MEACHAM, *J. A. M. A.*, **119**, 927, 1942.
- (5) JASPER, CONE, PRUDENZ AND BENNETT, *Surg. Gynecol. and Obstet.*, **76**, 599, 1943.
- (6) BRENNER AND COHEN, *J. A. M. A.*, **123**, 948, 1943.
- (7) GLAUBACH, *Proc. Soc. Biol. & Exp. Med.*, **42**, 325, 1939.

- (8) CLAUBACH, *Proc Soc Biol & Exp Med*, **46**, 53, 1941
- (9) ADRIANI, *J Lab & Clin Med*, **24**, 1066, 1939
- (10) BUTLER, DICKISON, COVIER, CRPER AND LAMSON, *THIS JOURNAL*, **72**, 208, 1941
- (11) CLAUBACH, to be published
- (12) MACKENZIE, MACKENZIE AND MCCOLLUM, *Science*, **94**, 518, 1941
- (13) ASTWOOD, SULLIVAN, BISSEL AND TYSLOWITZ, *Endocrinology*, **32**, 210, 1943
- (14) FRANKLIN AND CHAIKOFF, *J Biol Chem*, **148**, 719, 1943
- (15) MANA AND KEILIN, *Nature*, **146**, 164, 1940
- (16) HANZLIK AND CUTTING, *Science*, **98**, 389, 1943

THE TOXICITY AND TREPONEMICIDAL ACTIVITY OF AMIDE-SUBSTITUTED PHENYL ARSENOXIDES AND THEIR DERIVATIVES

HARRY EAGLE, RALPH B. HOGAN, GEORGE O. DOAK AND
HARRY G. STEINMAN¹

From the Venereal Disease Research and Postgraduate Training Center of the U. S. Public Health Service, Johns Hopkins Hospital, Baltimore—5, Maryland

Received for publication February 14, 1944

In a previous paper of this series (1) we have shown that acidie substituent groups regularly and markedly decreased the treponemicidal activity of phenyl arsenoxide (*T. pallidum*) without a commensurate decrease in toxicity. However, when the acidie group was blocked, as in ethyl or methyl esters, or as in the sulfone and phenone compounds, the treponemicidal activity was largely restored, and the ratio of treponemicidal activity:toxicity was increased as much as fourteen-fold, in several cases significantly exceeding that of the parent phenyl arsenoxide.

In the light of that finding, a series of phenyl arsenoxides was prepared in which an acidie substituent group had been blocked by amide formation (2). As will be shown in the present paper, the majority of these amides proved to be actively treponemicidal, and relatively low in toxicity. The ratio of treponemicidal activity:toxicity, which may be taken as a rough measure of potential therapeutic utility, was usually two to six times greater than that of the parent phenyl arsenoxide; and in the treatment of rabbit syphilis some of these compounds have shown a chemotherapeutic index approaching that of Mapharsen. This favorable effect of amide-substitution has been so regular as to suggest that further study may disclose other related compounds of greater therapeutic utility than those here described.

METHODS AND MATERIALS. *Compounds studied.* Of the 38 arsenoxides included in this series, seven were known compounds, prepared by methods indicated in the footnotes to table 1. The preparation of the 31 new compounds has been described elsewhere (2). Seven other amides (the o -CONH₂, o -SO₂NH₂, p -CONHC₆H₄CONH₂(p'), p -NHCH₂CONHCONH₂, p -CONHCONHCONH₂, p -CONHCH₂CONHCH₂CONH₂ and p -CH₂CONHCONH₂ phenyl arsenoxides) and six substituted amides (the p -CONH₂NH₂, p -CONH \cdot HNCOC₆H₄(p'), p -SO₂NHC₆H₄N, p -SO₂NHC₆H₄NS, p -CONHCH₂COOCH₃, and p -CONHCH₂CHOHCH₂OH phenyl arsenoxides) were found to hydrolyze to a marked degree when dissolved in dilute alkali preparatory to testing, and are not included in the tables. Four other compounds (the p -C₆H₄CONH₂(p'), p -NHCOC₆H₄CONH₂(p'), p -N=NC₆H₄CONH₂(p') and p -N=NC₆H₄CONHC₆H₄OH(p') phenyl arsenoxides) could not be tested because of their low solubility at pH 7.0.

Method of assay. The methods used for the assay of toxicity in white mice and rabbits, treponemicidal activity *in vitro* (cf. footnote,²), and therapeutic activity in syphilitic rab-

¹ With the technical assistance of Ralph Fleischman.

² Cf. (10) for preliminary report on the toxicity and *in vitro* treponemicidal activity of most of these compounds.

bits have been described in a preceding paper (3). In the latter assay the criterion of cure first used was the result of a popliteal lymph node transfer into the testis of a normal animal carried out six weeks after treatment. However, as has been shown in a preceding paper (4), a negative lymph node transfer at that time is not a reliable indication of cure in that a second lymph node transfer carried out six months after treatment is frequently positive. The curative doses given in the present paper are therefore based on the results of lymph node transfers carried out six months after treatment. In determining the dose which cured fifty and ninety five per cent of the animals for six months at least six rabbits were treated at each of 1 to 6 dosage levels.

EXPERIMENTAL RESULTS *A Toxicity and Direct Treponemicidal Activity of Amide Substituted Phenyl Arsenoxides*² As shown in table 1 substituents with terminal amide groups (CONH_2 , SO_2NH_2) regularly and markedly decreased the toxicity of phenyl arsenoxide, this regardless of the number and nature of the groups interposed between the amide group and the phenyl ring. Thus, the molar toxicity of the 16 amides listed in the Table based on the LD_{50} values, varied between 3.2 and 13.5 per cent that of the unsubstituted phenyl arsenoxide (column 3). A quantitatively similar detoxifying effect of amide substituents was observed in rabbits (table 3). Of the two disubstituted compounds included in table 1, the 3- NH_2 -4- CONH_2 phenyl arsenoxide was even less toxic than the simple p- CONH_2 compound, while the 3-OH-4- CONH_2 was more than twice as toxic.

The direct treponemicidal activity was also reduced by the amide substituents (cf. column 5 of table 1) but not to the same degree as toxicity, varying between 11 and 52 per cent that of the unsubstituted phenyl arsenoxide. In consequence, the ratio of treponemicidal activity to toxicity was favorably affected, and varied between two to six times that of the unsubstituted reference compound.

B The Effect of Substitution in the Amide Group The favorable effect of amide groups on toxicity was usually decreased, and in some cases obliterated if one or both of the terminal amide hydrogens were replaced by some substituent group. Thus, of 20 compounds derived from the p- SO_2NH_2 and p- CONH_2 phenyl arsenoxides, and listed in table 2, the toxicity was increased in 13, in several instances more than twenty fold. The treponemicidal activity was in such cases also increased but not to the same degree (cf. column 5). The ratio of activity to toxicity was therefore decreased, in some cases to one fifth its original high level. In this group of compounds the activity and toxicity of the compound had thus reverted toward that of the simple unsubstituted phenyl arsenoxide, or of phenyl arsenoxides with such "indifferent" substituents as CH_3 , Cl or OCH_3 groups (5). The only exceptions to this unfavorable effect of blocking the amide group were the compounds with terminal acetamido, nitrile and hydroxyl groups discussed in a following section.

C Toxicity and Therapeutic Activity in Rabbits Nine of the amides and three of the substituted amides in the present series of compounds were assayed for toxicity and therapeutic activity in rabbit syphilis. The results are given in table 3. The pronounced detoxifying effect of amide groups on phenyl arsenoxide is again evident. Although all the compounds were two to three times as toxic in rabbits as they were in mice, their relative toxicity was satisfactorily

TABLE 1

The toxicity and in vitro treponemoidal activity of amide-substituted phenyl arsenoxides

SUBSTITUENT GROUP INTRODUCED INTO PHENYL ARSENOXIDE	TOXICITY IN WHITE MICE ¹ (INTRAPERITONEAL INJECTION)			RELATIVE TREPO- NEMICIDAL ACTIVITY PER MOLE (I.E., PER GRAM AS) REFERRED TO THAT OF PHENYL ARSENOXIDE AS 100	RATIO OF TREPONEMICIDAL ACTIVITY IN VITRO ² TOXICITY IN WHITE MICE REFERRED TO THAT OF PHENYL ARSENOXIDE AS 1
	"Maximal tolerated dose" (LD ₅₀)	LD ₅₀	Molar toxicity referred to that of phenyl arsenoxide as 100		
	mg./kg.	mg./kg.			
Unsubstituted phenyl ar- senoxide .	1.5	1.93	100.0	100	1
3-NH ₂ -4-OH (Maphar- sen)	33.5	42.6	6.94	38	5.5
m-CONH ₂ ²	17	24.6	9.8	41	4.1
p-CONH ₂ ^{2,3}	17	27.5	9.6	45	4.6
p-CH=CHCONH ₂	20	28	9.7	43	4.4
p-CH ₂ CONH ₂	24	30	8.6	20	2.3
p-(CH ₂) ₃ CONH ₂	15	21.6	13.5	33	2.4
p-CONHCONH ₂ ³	40	48.4	6.4	34	5.2
p-CONHCH ₂ CONH ₂	67	79.5	3.86	24	6.1
p-CONHCH ₂ CH ₂ CONH ₂	60	100	3.2	13	4.1
p-CH ₂ CONHCH ₂ CONH ₂ ³	90±	103	3.4	11	3.15
p-OCH ₂ CONH ₂ ³	17	33	9.0	52	5.7
p-NHCONH ₂ ³	24	35	8.1	38	4.7
p-NHCH ₂ CONH ₂ ⁴	42	61	4.5	22	4.8
p-NHCO(CH ₂) ₂ CONH ₂	24	36	9.0	25	2.7
m-SO ₂ NH ₂	36	46.4	6.1	21	3.5
p-SO ₂ NH ₂ ³	42	63	4.8	29	6.1
p-SO ₂ NHCH ₂ CONH ₂	64	99	3.5	17	5.05
3-NH ₂ -4-CONH ₂	35	47	5.6	23	5.0
3-OH-4-CONH ₂	9.5±	12	23	45	1.93

¹ All values given in this table are calculated from the experimental data by the Reed-Muench procedure (13), extrapolating where necessary to obtain the LD₅₀ and LD₁₀₀ values. For each compound, 8 to 20 mice were used at each of 4 to 6 dosage levels, and were followed for 4 days before being adjudged as dead or survived. Although the values for minimal lethal dose (LD₁₀₀), are not given in the Table, they averaged twice the maximum tolerated dose (LD₅₀), with the LD₁₀₀ approximately a geometric mean between the two.

² Prepared following the directions of Gough and King (J. Chem. Soc., 1930, p. 669).

³ Isolated as the arsonoso compound, -As(OH)₂, rather than the arsenoso, -AsO. Whenever both forms have been prepared they have proved to be identical in biological activity per mole.

⁴ Prepared following the directions of Cohen, King, and Strangeways (J. Chem. Soc., 1932, p. 2505).

⁵ The experimental values in the preceding two columns have been given to the nearest whole number. In computing the ratio of activity:toxicity, the actual experimental values have been used.

concordant in the two species (cf. column 4 of tables 1, 2 and 3). By virtue of that decreased toxicity, all the amides tested gave favorable values for the ratio of LD₅₀ (dose which killed half of animals)/CD₅₀ (dose which cured half of

animals), which may be taken as a measure of therapeutic utility ("chemotherapeutic index") In the case of mapharsen that value was 13, and the cor-

TABLE 2

The effect of substitution in the amide group on the toxicity and direct treponemicidal activity of p CONH₂ and p SO₂NH₂ phenyl arsenoxides

SUBSTITUENT GROUP INTRODUCED INTO PHENYL ARSENOXIDE	TOXICITY IN WHITE MICE ¹ (INTRAPERITONEAL INJECTION)			RELATIVE TREP- ONEMICIDAL ACTIVITY PER MOLE (I.E. PER GRAM As) REFERRED TO THAT OF PHENYL ARSENOXIDE AS 100	RATIO OF TREPONEMICIDAL ACTIVITY IN VITRO TOXICITY IN WHITE MICE REFERRED TO THAT OF PHENYL ARSENOXIDE AS 1
	M ₅₀ maltol- erated dose (LD ₅₀)	LD ₅₀	Molar toxicity referred to that of phenyl arsenoxide as 100		
	mg/kg	mg/kg			
p-SO ₂ NH ₂	42	63	4.8	20	6.1
p-SO ₂ NHCH ₃	10	16.9	17.7	72	4.0
p-SO ₂ N(CH ₃) ₂ ²	2.55	3.61	92.5	112	1.2
p-SO ₂ NHC ₂ H ₅	8.05	10	31.8	72	2.3
p-SO ₂ N(C ₂ H ₅) ₂ ³	2.2	2.75	134	101	0.74
p-SO ₂ NHC ₂ H ₄ OH ³	71	83.7	4.25	23	5.3
p-CONH ₂ ¹	17	27.5	9.6	45	4.6
p-CONHCH ₃ ²	10	17.2	15.0	54	3.6
p-CON(CH ₃) ₂	9.6	14.3	19	48	2.5
p-CONHC ₂ H ₅ ²	8.8	10.6	26	59	2.3
p-CON(C ₂ H ₅) ₂	3.8	4.8	64	53	0.84
p-CONHC ₂ H ₄ ²	2.6	3.44	101	97	0.96
p-CONHCH ₂ C ₂ H ₅	3	4.3	80	70	0.08
p-CONHC ₂ H ₄ N[(C ₂ H ₅) ₂ CH] benzamide]	2.3	2.8	116	74	0.64
$\begin{array}{c} \text{NH} \\ \\ \text{p}-\text{C} \\ \\ \text{OC}_2\text{H}_5 \end{array}$	2.9	3.5	87	68	0.78
p-CONHC ₂ H ₄ COOH ²	14	21	15.7	0.7	0.04
p-CONHC ₂ H ₄ NHCOCH ₃ (p)		200±	1.0±	9.0	4.5
p-CONHCH ₂ CH ₂ NHCOCH ₃ ²	60	95	3.6	16	4.4
p-CONHC ₂ H ₄ OH ²	51	64.3	4.8	25	5.2
p-CONHCONHC ₂ H ₄ OH	43	68	5.0	30	6.0
p-CONHCH ₂ CHOHCH ₂ OH	74	80	3.7	14	3.7
p-CONHCH ₂ CN	40	63	4.5	27	6.0

¹ Cf footnote 1 in table 1

² Cf footnote 2 in table 1

³ Cf footnote 3 in table 1

⁴ Prepared following the directions of Hugounenq L. and Morel, A (J de Pharmacie et de chim., 7 (7) 383 (1913))

responding figures for the ten amide substituted compounds tested in rabbits varied between 17 and 37

As previously noted, substitution in the amide group with "indifferent" substituents such as -C₂H₅ or -C₆H₅ groups caused a marked increase in toxicity,

and a resultant decrease in the ratio of activity/toxicity. On the other hand, when one of the amide hydrogens was replaced with a group containing a terminal hydroxyl the resulting compounds did not differ significantly from the parent amide with respect to toxicity, and the therapeutic activity, like the treponemicidal action *in vitro*, was decreased rather than increased.

The validity of the *in vitro* assay of treponemicidal activity as a first approximation of therapeutic activity was confirmed by the close correlation between the two, graphically shown in figure 1. In these phenyl arsenoxides, as for those

TABLE 3

The toxicity in rabbits and therapeutic activity in rabbit syphilis of amide-substituted phenyl arsenoxides and their derivatives

COMPOUND (R ₁ C ₆ H ₄ AsO or R ₁ R ₂ C ₆ H ₃ AsO)	TOXICITY IN RABBITS ¹			THERAPEUTIC ACTIVITY			LD ₅₀ CD ₅₀
	Maximal tolerated dose	LD ₅₀	Molar toxicity referred to that of phenyl arsenoxide as 100	CD ₅₀		Minimal curative dose (CD> 95)	
				Mg./kg.	Mg./ As/kg.		
	mg./kg.	mg./kg.				mg./kg.	
Phenyl arsenoxide . .	0.59	0.79	100	No cures in sublethal doses			<1
3-NH ₂ -4-OH . .	10.0	13.0	9.3	3.0	0.9	6.3	4.3
m-CONH ₂	5.5	7.3	13.6	2.2	0.78	3.8	3.3
p-CONH ₂	6.0	9.1	11.9	2.8	0.92	6±	3.3
p-CH ₂ CONH ₂	7.8	9.4	11.2	4.5(?)	1.5(?)	>6	2.1±
p-CH=CHCONH ₂	7.5	9.5	11.9				
p-CONHCH ₂ CONH ₂	16.0	24.0	5.2	15	4.2		1.7
p-OCH ₂ CONH ₂ . .	9.8	10.5	11.6	3.8	1.1	6.5	2.8
p-NHCONH ₂ . .	8.5	11.7	9.8	4.6	1.4	10	2.5
p-SO ₂ NH ₂	12.5	16.1	7.7	6.	1.72	11	2.7
p-SO ₂ NHCH ₂ CONH ₂	13	25.	5.8	25±	4.5±		1±
3-NH ₂ -4-CONH ₂	7.5	13.6	8.7	3.7	1.2	7.5	3.7
p-CONHC ₂ H ₅ . .	2.5	3.9	28.9				
p-CONHC ₂ H ₅	1±	1.7±	83±	No cures in sublethal doses			<1
p-CONHC ₂ H ₄ OH	15.5	19.6	6.1	10	2.8	16	2.0
p-SO ₂ NHC ₂ H ₄ OH	11.6	16.5	8.8	11	2.65	16	1.5

¹ Minimal lethal dose not indicated in table, but averaged 50 per cent more than the LD₅₀ value.

² Inexact: Internal conflict in results at different dosages.

previously reported, the direct treponemicidal activity is apparently the primary factor in determining therapeutic activity. Such other factors as varying rates of excretion, or conversion *in vivo* to compounds either more or less active than that injected, are apparently of secondary importance.³

³ The recent failure of Kolmer, Kast and Rule (6) to obtain consistent results in their *in vitro* assays of treponemicidal activity rests in part on the fact that they were working with arspenamines. These are known to be inactive as such (7), and must first be oxidized, presumably to the corresponding arsenoxide. In the original description of the *in vitro* assay (3) it was stressed that the method is applicable only to those compounds which, like phenyl arsenoxides, act directly on the spirochete, without necessary preliminary

DISCUSSION The foregoing results indicate a definite correlation between the chemical structure and biological activity of amide substituted phenyl arsenoxides and their derivatives. Terminal amide groups ($R\text{-CONH}_2$, $R\text{SO}_2\text{NH}_2$) regularly caused a marked (80 to 90 per cent) decrease in the toxicity of phenyl arsenoxide (table 1), but did not decrease its treponemicidal activity to the same degree (table 1). In consequence, the ratio of treponemicidal activity to toxicity was 1.9 to 6.1 times as favorable as that of the parent phenyl arsenoxide. Similarly favorable chemotherapeutic indices were obtained on determining the toxicity and therapeutic activity of these compounds in syphilitic rabbits.

This detoxifying effect was a property of terminal amide groups, and was observed whether that amide group was attached directly to the benzene ring, as in the case of the 3- and 4- CONH_2 and $\text{-SO}_2\text{NH}_2$ compounds, or through some intermediate linkages, as in the substituents -NHR , $\text{-OCH}_2\text{R}$, -CH=CHR , $\text{-CH}_2\text{R}$, $\text{-(CH}_2)_3\text{R}$, $\text{-NHCO(CH}_2)_2\text{R}$, and $\text{-NHCH}_2\text{R}$, where R stands for the -CONH_2 grouping. The only amides so far encountered in which this favorable effect was not observed were compounds found to hydrolyze in dilute alkali to the corresponding acid (1).

When either or both of the amide hydrogens were substituted, the chemotherapeutic properties of the compound shifted toward those characteristic

conversion to other compounds. It should further be pointed out that Kolmer, Knst and Rule failed to control adequately some of the important variables in the *in vitro* assay.

a Time factor As shown in an earlier paper (7), the spirocheticidal (actually, spirochete-immobilizing) activity of arsenicals *in vitro* is a function of time. In concentrations comparable to those momentarily attained in the human body after their therapeutic administration, arsphenamines and arsenoxides usually have no visible effect for a variable period, sometimes exceeding one hour. Only after this initial lag does one observe the characteristic progressive immobilization. In the fifteen minute incubation period used by Kolmer and his co-workers, only relatively large concentrations of arsenical would have an effect, and it follows from the shape of the time immobilization curves (7) that in those high concentrations, with rapid and progressive immobilization during the counting period, quantitative comparisons would be difficult if not impossible. Even with the longer exposure time (two to four hours) used in our own experiments, it has been necessary to control this variable by the addition of cysteine, which instantaneously stops the action of the arsenoxides (8).

b Aerobiosis Suspensions of spirochetes obtained from rabbit chancres tend to become progressively less motile if kept aerobically at room temperatures. We have regularly found the spirochete immobilizing activity of the phenyl arsenoxides to be greater aerobically than anaerobically, probably because aerobically there is a summation of the deleterious effects of oxygen and of arsenical. To control this variable we have found it necessary to conduct our assays in an anaerobe jar, under hydrogen, and to add cysteine to all the tubes immediately after their removal from the jar preparatory to counting.

c Tissue extractive Another important factor which Kolmer and his associates have failed to control adequately is the fact that tissue extractives may modify the spirochete-immobilizing activity of arsenicals (and of bismuth) to an extraordinary degree (7) (8) (9). Uncontrollable variations in the amount of tissue extractives in emulsions prepared in the same manner from different chancre testes may produce large differences in the apparent activity of an arsenoxide. It follows that the *in vitro* assay cannot be absolute, but that the activity of an unknown compound must in every instance be compared with that of some reference compound tested at the same time, under the same conditions, and with the same spirochetal suspension.

of the new terminal substituent. Thus, the simple 4-CH₃ phenyl arsenoxide had previously been shown (5) to have the same treponemicidal activity as phenyl arsenoxide, an even greater toxicity, and an activity/toxicity ratio of $102/121 = 0.83$, referred to that of phenyl arsenoxide as $100/100 = 1$. The corresponding index for the 4-SO₂NH₂ compound was $29.1/4.8 = 6.1$. When one of the hydrogens of the p-SO₂NH₂ compound was replaced with a -CH₃ group both activity and toxicity were increased, and the original index of 6.1

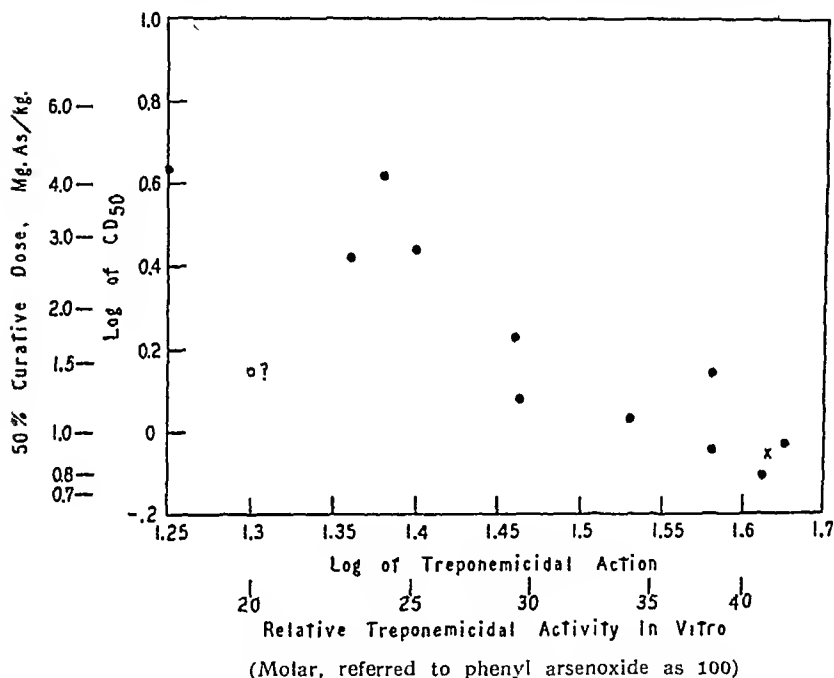


FIG. 1. THE CORRELATION BETWEEN THE TREPONEMICIDAL ACTIVITY IN VITRO AND THERAPEUTIC ACTIVITY IN SYPHILITIC RABBITS OF A SERIES OF PHENYL ARSENOXIDES

- = Amide-substituted compounds and their derivatives listed in table 3.
- × = Mapharsen.
- = Questionable value for p-CH₂CONH₂ compound.

fell to $71.5/17.7 = 4.0$. When both amide hydrogens were replaced with -CH₃ groups, the activity/toxicity ratio fell to $112/92.5 = 1.2$. Clearly, the properties of the compound had shifted toward those of the new terminal -CH₃ groups (p-SO₂N(CH₃)₂). Substituting one or both amide hydrogens with -C₂H₅ groups had the same effect. In the corresponding four -CONH₂ derivatives, a similar effect was produced by -CH₃ and -C₂H₅ substitution.

A benzyl or phenyl group substituted in one of the -CONH₂ hydrogens also obliterated the favorable effect of the amide. The most striking example of the fact that the properties of the substituted amides are determined in large part

by the terminal substituent was afforded by the $p\text{-CONHCH}_2\text{COOH}$ phenyl arsenoxide. Acid substituents are known to cause a marked decrease in treponemicidal activity (1); and the molar activity of this compound was 0.68, as compared with 44.5 for the unsubstituted amide, both values referred to that of phenyl arsenoxide as 100. The toxicity was at the same time slightly increased, giving an activity/toxicity ratio of $0.68/15.7 = 0.04$, less than $\frac{1}{16}$ that of the original amide.

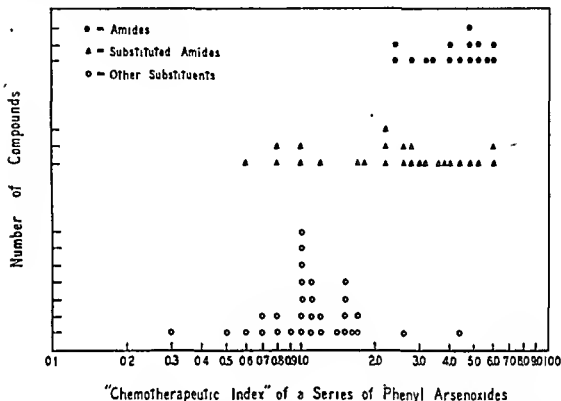


FIG. 2. THE "CHEMOTHERAPEUTIC INDEX" OF AMIDE-SUBSTITUTED PHENYL ARSENOXIDES AND THEIR DERIVATIVES, COMPARED WITH A VARIETY OF OTHER MONO-SUBSTITUTED PHENYL ARSENOXIDES

The chemotherapeutic index plotted in the figure is the ratio of *treponemicidal activity in vitro*: toxicity in white mice, in each case referred to that of the unsubstituted phenyl arsenoxide as 1.0.

Of those compounds in which substitution in an amide group did not obliterate or impair its favorable effect, four had terminal hydroxyl groups ($p\text{-CONHC}_2\text{H}_4\text{OH}$, $p\text{-SO}_2\text{NHC}_2\text{H}_4\text{OH}$, $p\text{-CONHCONHC}_2\text{H}_4\text{OH}$, and $p\text{-CONHCH}_2\text{CHOHCH}_2\text{OH}$) and two others had terminal acetamido groups ($p\text{-CONHC}_6\text{H}_4\text{NHCOCH}_3$ and $p\text{-CONHCH}_2\text{CH}_2\text{NHCOCH}_3$). It is significant that both of these terminal groupings, unlike any of those discussed in the preceding paragraphs, are known to exert a favorable chemotherapeutic effect if substituted directly into phenyl arsenoxide (cf. (5)), due primarily to the decreased toxicity of the substituted compound.

The generally favorable effect of amide substitution on the ratio of treponemicidal activity:toxicity, compared with that of other substituents (e.g., $-\text{CH}_3$,

$-C_2H_5$, $-NO_2$, $-NH_2$, $-OH$, $-NHCOCH_3$), and the intermediate position in that respect occupied by substituted amides, are graphically summarized in figure 2.

It has recently been shown (11) that the toxicity of phenyl arsenoxides is primarily a function of the degree to which they are bound by tissues. The low toxicity of the amide-substituted compounds is therefore probably related to the fact that they are not bound by the body cells to the same degree as the unsubstituted compound, or arsenoxides with e.g., $-CH_3$, $-C_2H_5$, or $-NO_2$ groups. Whether this rests on a selective permeability of the cell membrane, or on the varying affinity of these compounds for cellular constituents which, like $-SH$ containing enzymes, are inactivated by arsenicals (12), are points which are now under study.

SUMMARY

Thirteen amide-substituted phenyl arsenoxides ($-RCONH_2$, $-RSO_2NH_2$) were, per unit arsenic, only 4.5 to 13.5 per cent as toxic as the parent phenyl arsenoxide. Since the treponemicidal activity *in vitro* was not reduced to the same degree, the ratio of treponemicidal activity:toxicity was 1.9 to 6.1 times more favorable than that of phenyl arsenoxide. The favorable effect of amide groups was confirmed for ten of these compounds by assays of toxicity and therapeutic activity in syphilitic rabbits.

When one or both of the amide hydrogens were substituted (e.g., $-SO_2N(CH_3)$, $-CONH$ -pyridine), the effect of the entire group shifted toward that of the terminal substituent. In most cases, substitution in the amide therefore caused an increased toxicity, and impaired the favorable effect of the amide group as such. Only in the case of the compounds with terminal hydroxyl acetamido or nitrile groups was the favorable effect of the amide altogether preserved, perhaps because these groups in themselves depress the toxicity of phenyl arsenoxide.

The regularity with which substituents containing terminal amide groups decreased the toxicity and increased the chemotherapeutic index of phenyl arsenoxide suggests that some members of this series may be of clinical utility.

REFERENCES

- (1) EAGLE, HOGAN, DOAK, AND STEINMAN, THIS JOURNAL, 70: 221, 1940.
- (2) DOAK, STEINMAN, AND EAGLE, J. A. C. S., 62: 168, 1940; *ibid.*, 62: 3012, 1940; *ibid.*, 63: 99, 1941; *ibid.*, 66: 194, 1944. STEINMAN, DOAK, AND EAGLE, J. A. C. S., 66: 192, 1944. DOAK, EAGLE, AND STEINMAN, J. A. C. S., 62: 3010, 1940.
- (3) EAGLE, THIS JOURNAL, 69: 342, 1940.
- (4) EAGLE, HOGAN, AND KEMP, Am. J. Syph., 26: 557, 1942.
- (5) EAGLE, DOAK, HOGAN, AND STEINMAN, THIS JOURNAL, 70: 211, 1940; *ibid.*, 70: 221, 1940.
- (6) KOLMER, KAST, AND RULE, Am. J. Syph., 24: 201, 1940.
- (7) EAGLE, THIS JOURNAL, 64: 164, 1938; *ibid.*, 66: 423, 1939.
- (8) EAGLE, THIS JOURNAL, 66: 436, 1939.
- (9) EAGLE, Am. J. Syph., 23: 310, 1939.
- (10) EAGLE, HOGAN, DOAK AND STEINMAN, J. A. C. S., 65: 1236, 1943.
- (11) HOGAN AND EAGLE, THIS JOURNAL, 80: 93, 1944.
- (12) BARRON AND SINGER, Science, 97: 356, 1943.
- (13) REED AND MUENCH, Am. J. Hyg., 27: 493, 1938.

PHARMACOLOGY AND CHEMISTRY OF SUBSTANCES WITH CARDIAC ACTIVITY

III THE EFFECT OF SIMPLE UNSATURATED LACTONES AND *t* BUTYL HYDROGEN PEROXIDE ON THE ISOLATED FROG HEART¹

RAFAEL MENDEZ

From the Department of Pharmacology Harvard Medical School, Boston

Received for publication February 16, 1944

In previous communications from this laboratory it was shown that simple unsaturated lactones like the angelicalactones (1), as well as ascorbic acid (2), cause systolic standstill of the isolated frog heart and counteract the effect of a perfusion fluid poor in calcium ions. In this regard the substances are similar in their action to the cardiac glycosides which contain in their molecule an unsaturated lactone ring.

Krayer found that the action of ascorbic acid is not due to this substance itself but can be accounted for by the formation of hydrogen peroxide during the process of dehydrogenation occurring in solutions of ascorbic acid unless they are adequately protected (2, 3). Hydrogen peroxide itself (2), the peroxide formed during the autoxidation of diethylether (4), and benzoyl hydrogen peroxide (5) cause systolic standstill of the isolated frog heart.

That hydrogen peroxide was responsible for the action of ascorbic acid was shown (2, 3) by preventing the cardiac effect of ascorbic acid solution in two ways: 1) by facilitating the destruction of hydrogen peroxide so that in spite of continuous formation the concentration stayed below the level required for the effect upon the heart, 2) by preventing the formation of hydrogen peroxide. The first was achieved by the use of catalase and of sodium pyruvate, the latter was accomplished by the use of serum globulin and diethyl-dithiocarbamate, substances which form complexes with copper and thereby inhibit copper catalysis, this having been shown to play an important role in the dehydrogenation of ascorbic acid (6).

The elucidation of the action of ascorbic acid made it necessary to examine whether the unsaturated lactones found active on the frog heart also owed their action to the formation of substances with the characteristics of peroxides. For this purpose it was investigated whether peroxides could be detected in the solutions of α,β and β,γ angelicalactone, and to what extent the effect of the angelicalactones upon the frog heart could be modified by two groups of substances: (1) catalase, sodium pyruvate, and peroxidase, (2) serum globulin, cysteine, glutathione, and diethyl-dithiocarbamate.

As it was possible to secure a stable water soluble organic peroxide, *t* butyl hydrogen peroxide, the biological action of which had not been studied hitherto,

¹ This work was carried out under the auspices of the University Committee on Pharmacotherapy.

the cardiac effect of this substance was compared with that of the angelicalactones and of ascorbic acid.

The α,β - and β,γ -angelicalactone used in this work were prepared by R. P. Linstead and D. Todd as previously described (1). The sample of *t*-butyl hydrogen peroxide was supplied by N. A. Milas, according to whose estimations it contained 98% $(\text{CH}_3)_3\text{C}-\text{O}-\text{O}-\text{H}$ and had approximately 16% available active oxygen; it was a colorless fluid with a specific gravity of 0.91 (7).

METHODS. The experiments were carried out on the isolated hearts of male frogs of the species *Rana pipiens* during all months of the year, using the cannula previously described (2), which allowed continuous replacement of the physiological salt solution. The rate of replacement was uniformly kept at approximately 2 cc. per minute. The physiological salt solution had the following composition: NaCl 0.65%; KCl 0.014%; CaCl_2 (anhydrous) 0.011%; NaHCO_3 0.02% (Clark's Solution). The pH of the solution was approximately 8.0. The experiments were carried out at room temperature between 23 and 26°C. unless otherwise mentioned. Appropriate oxygenation of the solution was maintained by bubbling a stream of air through the fluid in part A of the cannula.

For the estimation of the presence of peroxide and of differences in the peroxide concentration of the solutions, Lommel's reagent was used as previously described (2). To prepare the reagent for the present study, hemin was employed, as no mesohemin was available; 100 mgm. of 3-aminophthal hydrazide ("luminol") and 5 mgm. of hemin were made up to 100 cc. with a solution of 1% anhydrous sodium carbonate. Five cc. of this reagent were mixed with 10 cc. of the solution to be tested. The observations were made in the dark-room, after thorough adaptation of the eyes and with the test tube at a distance of 50 cm. from the eye of the observer.

The catalase used was a solution containing some suspended particles of a highly purified sample of crystalline beef liver catalase supplied by J. B. Sumner; the peroxidase was prepared from milk by the method of Elliott (8), or from horseradish by the method of Keilin and Mann (9); the serum globulin was obtained from the laboratory of E. J. Cohn and consisted of 90% γ - and 10% β -globulin. The dilutions of catalase refer to the solution of Sumner's preparation, 0.05 cc. of which added to 500 cc. ($= 1 \times 10^{-4}$) was shown to be able to prevent the cardiac effect of a solution of *l*-ascorbic acid 1:10,000 at pH 7.5 to 7.8, in the presence of oxygen (2). Unless otherwise stated, the concentration of all other substances mean weight in grams per volume in cc. of Clark solution. The concentration of the angelicalactones and of *t*-butyl hydrogen peroxide in the test with 3-aminophthal hydrazide refer to the dilution in Clark solution used for the test and not to the concentration in the final reaction mixture. In preparing the solutions for the study of the modifying effect of the two groups of substances upon the cardiac action of the lactones and *t*-butyl hydrogen peroxide, the potential modifying agent (e.g., sodium pyruvate) was first dissolved in the Clark solution in the desired concentration before the angelicalactone or *t*-butyl hydrogen peroxide was added.

I. CARDIAC ACTION AND PEROXIDE CONTENT OF THE ANGELICALACTONE SOLUTIONS. The solutions of α,β - and β,γ -angelicalactone in concentrations capable of a heart effect contained peroxides, as could be shown by the reaction of the solutions with 3-aminophthal hydrazide. The intensity of the luminescence was proportional to the concentration of the lactones and in equimolar solutions was much stronger with β,γ -angelicalactone than with α,β -angelicalactone. The minimal concentration of β,γ -angelicalactone at which luminescence was distinct was one part in 3 to 5 million, while for α,β -angelicalactone the dilution was one part in 200,000 to 300,000.

In its ability to cause systolic arrest of the isolated frog heart β, γ angelicalactone was found to be about ten times more potent than α, β angelicalactone (1). The difference in the luminescence reaction of the two lactones therefore corresponds to the difference in their cardiac activity.

Perfusion of the solutions of the angelicalactones through the heart did not noticeably alter the intensity of the luminescence, this was of about the same strength in samples of the same solution before and after contact with the heart muscle tissue.

The substances capable of destroying hydrogen peroxide or of interfering with peroxide formation did not modify in a uniform way the cardiac action of the angelicalactone solutions. The varied results are illustrated in figure 1 for the α, β angelicalactone.

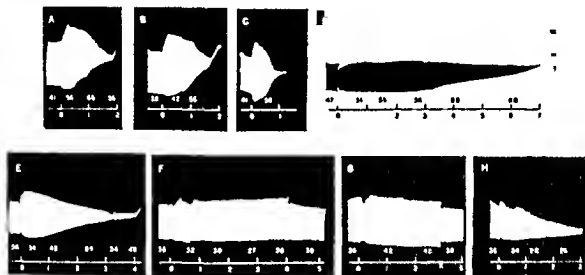


FIG. 1. EFFECT OF α, β ANGELICALACTONE (2×10^{-4}) ON THE ISOLATED FROG HEART.

The administration of the lactone solution or of the lactone solution with the added substance started at zero time. At X in G, the drum was stopped for 20 mins and 30 minutes.

A. Effect of catalase, peroxidase, and sodium pyruvate. While hydrogen peroxide readily reacts with catalase, peroxidase, and sodium pyruvate, the peroxides present in the solutions of the angelicalactones were to a greater or lesser degree resistant to these agents. It can be seen from table 1 that a concentration of catalase as high as 2×10^{-4} of Sumner's preparation of crystalline catalase exerted very little if any protection against the action of β, γ angelicalactone upon the heart and had no effect on the action of α, β angelicalactone. Peroxidase in a concentration of 2×10^{-4} inhibited or at least greatly delayed the action of β, γ angelicalactone, while it did not protect the heart against the effect of α, β angelicalactone. A difference similar to that between the protective action of catalase and peroxidase on the β, γ angelicalactone was noticed by Sumner (10) with regard to the water soluble peroxide formed by exposing oil

TABLE 1

The effect of catalase, peroxidase, and sodium pyruvate on the action of β , γ - and α , β -angelicalactone and *t*-butyl hydrogen peroxide on the isolated frog heart

SUBSTANCE	CONCENTRATION	NO OF EXPS	TIME TO CATALASE SYSTOLIC STANDSTILL minutes	EFFECT OF CATALASE			EFFECT OF PEROXIDASE			EFFECT OF SODIUM PYRUVATE		
				Concentration	No of expts	Heart action	Concentration	No of expts	Heart action	Concentration	No of expts	Heart action
β , γ -angelicalactone	1×10^{-3}	7	115-170	1×10^{-4}	3	Complete systolic effect in 141-150 minutes	1×10^{-3}	1	Complete systolic effect after 174 min	1×10^{-3}	4	No systolic effect after 3 hours
				2×10^{-4}	1	Complete systolic effect in 200 min	2×10^{-3}	1	Systolic effect started in about 120 minutes, complete after 322 minutes			
	2×10^{-3}	5	64-83				1×10^{-4}	2	No systolic effect after 200 minutes			
							2×10^{-4}	3	No systolic effect after 200 minutes			
α , β -angelicalactone	2×10^{-4}	7	74-100	2×10^{-4}	2	Complete systolic effect in 105-106 minutes	2×10^{-4}	1*	No systolic effect after 200 minutes	1×10^{-3}	2	Systolic effect after 3 hours, complete in about 7 hours
	1×10^{-4}	4	51-92	2×10^{-4}	1	Complete systolic effect in 70 minutes	2×10^{-4}	2*	Complete systolic effect after 65-79 minutes	1×10^{-3}	2	Systolic effect starts in approx 3 hours and is complete in approx 7 hours
<i>t</i> -butyl hydrogen peroxide	1×10^{-4}			2×10^{-4}			2×10^{-4}	1*	Complete systolic effect in 35 min (23°C)	1×10^{-3}	2	Complete systolic effect in 54-100 minutes

* These experiments were done with horse-radish peroxidase, the others with milk peroxidase.

of turpentine and water to oxygen and light. Sodium pyruvate 1×10^{-3} greatly delayed, although it did not prevent, the effect of both α, β and β, γ -angelicalactone, while α, β and β, γ angelicalactone both produced complete systolic effect in a period of approximately $1\frac{1}{2}$ hours in concentrations of 2×10^{-4} and 2×10^{-5} respectively, it took approximately 7 hours to observe the same effect in the presence of pyruvate (see fig 1, D, for α, β angelicalactone)

B Effect of serum globulin, cysteine, glutathione, and diethyl dithiocarbamate
As shown in table 2, cysteine and glutathione completely protected the heart against the action of α, β and β, γ angelicalactone for periods longer than 3 hours. Serum globulin also inhibited the effect of both angelicalactones when it was present in a concentration of 2×10^{-4} . A concentration of 1×10^{-4} considerably delayed the effect, for it took approximately 6 hours to cause complete systolic effect against $1\frac{1}{2}$ to 2 hours in the control experiments (see fig 1, A, and E). Diethyl dithiocarbamate 1×10^{-5} delayed or prevented the action of both lactones, but it could not be accurately ascertained to what degree because diethyl dithiocarbamate in this concentration in itself was capable of bringing about a systolic effect within a period of approximately 5 to 7 hours.

C The luminescence reaction with 3 aminophthal hydrazide The substances used (see A and B) to delay or prevent the action of the angelicalactones upon the frog heart did not affect in all cases to a corresponding degree the appearance and intensity of luminescence produced by the angelicalactone solutions when reacting with 3 aminophthal hydrazide. Catalase, when the concentration was high enough to cause a slight delay in the cardiac effect of β, γ angelicalactone (table 1), also slightly decreased the luminescence. Sodium pyruvate and diethyl dithiocarbamate distinctly diminished the intensity of the luminescence when used in concentrations that delayed or abolished the effect upon the frog heart. On the other hand serum globulin in concentrations capable of distinctly affecting the heart action of the angelicalactones did not noticeably decrease the luminescence. Cysteine or glutathione actually increased the intensity of the luminescence in the solutions of the angelicalactones when reacting with 3 aminophthal hydrazide. This can probably be explained by the fact that cysteine and glutathione are able to form peroxides when exposed to oxygen in strongly alkaline solution (11).

II EFFECT OF *t* BUTYL HYDROGEN PEROXIDE The characteristic effect of *t* butyl hydrogen peroxide on the frog heart mounted according to the Straub-Fühner technique is shown in figure 2. A concentration 2×10^{-3} produced a short lasting depressant effect followed by a period of half rhythm and then by an increase in amplitude with increase in heart rate, within a period of approximately 30 minutes the ventricle stopped in systole. When the perfusion fluid was continuously replaced, a concentration of 1×10^{-4} caused systolic standstill in a period of 54 to 92 minutes (see fig 3, A).

t Butyl hydrogen peroxide and β, γ angelicalactone had a cardiac activity of the same order, as can be seen from a comparison of the above results with those previously reported [(1), see fig 7]. The limit concentration of *t* butyl hydrogen peroxide to give a distinct luminescence with 3 aminophthal hydrazide was one

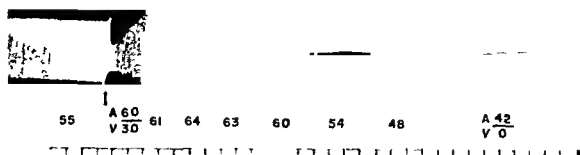


FIG. 2. EFFECT OF *t*-BUTYL HYDROGEN PEROXIDE ON THE ISOLATED FROG HEART. Straub technique. At sign, *t*-butyl hydrogen peroxide 2×10^{-2} . The numbers above the time line indicate heart rate per minute. A, auricle; V, ventricle. Time in minutes.

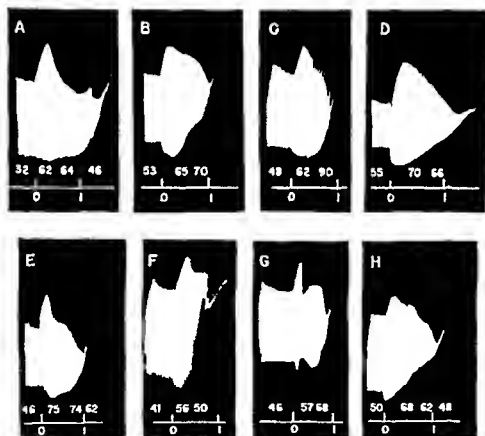


FIG. 3 EFFECT OF *t*-BUTYL HYDROPEROXIDE (1×10^{-4}) ON THE ISOLATED FROG HEART

fluid A, *t* butyl hydrogen peroxide
peroxidase 2×10^{-4} , D, with sodium
pyruvate 1×10^{-4} , G, with glutathione
25 1×10^{-4} . The numbers above the
time line indicate heart rate per minute. Time in hours. The administration of the
solution of *t* butyl hydrogen peroxide, or of the peroxide with the added substance, started
at zero time.

part in 2 to 3 million, and its activity in this regard, therefore, is also of approximately the same order as that of β,γ -angelicalactone, and, like the cardiac effect, about ten times greater than that of α,β -angelicalactone. This relation also holds on a molar basis, as the molecular weight of the angelical actonosis is 98, while that of *t*-butyl hydrogen peroxide is 90.

As shown in tables 1 and 2, none of the substances which destroy hydrogen peroxide or interfere with its formation and which delay or prevent the action of solutions of ascorbic acid or of the angelicalactones had any delaying or diminishing effect whatsoever on the action of *t*-butyl hydrogen peroxide upon the frog heart. From figure 3 it can be seen that the heart action also was not modified in a qualitative way by any of the substances administered together with the *t*-butyl hydrogen peroxide. This, of course, was to be expected, as the substances used interfere with the formation of the peroxides rather than with their biological action. Similarly, the luminescence with 3-aminophthal hydrazide produced by the solutions of *t*-butyl hydrogen peroxide was not inhibited to any extent by any of the substances mentioned.

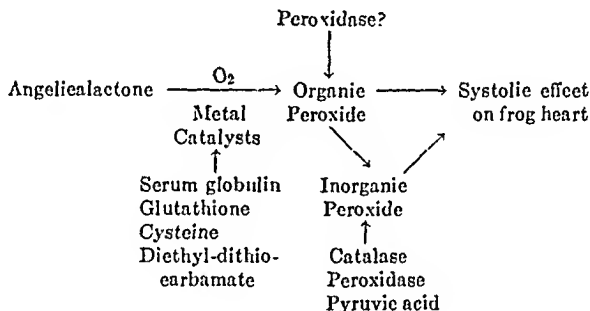


FIG. 4

SUMMARY AND CONCLUSIONS

The effect of α,β - and β,γ -angelicalactone on the isolated frog heart can be accounted for by the formation of peroxides in the solutions of these substances. The relative intensity of the cardiac activity of solutions of α,β - and β,γ -angelicalactone corresponds to the relative intensity of the luminescence occurring when the solutions react with 3-aminophthal hydrazide.

The peroxides of the angelicalactones are resistant to the action of catalase. Peroxidase reacts more readily with the peroxide of β,γ -angelicalactone than with that of its α,β -homologue. Sodium pyruvate reacts distinctly and about equally well with the peroxides of the two angelicalactones. Serum globulin, cysteine, glutathione, and diethyl-dithiocarbamate, the substances which form copper complexes and interfere with copper catalysis, greatly decrease or prevent altogether the effect of both angelicalactones upon the heart. The effect of these groups of substances upon the formation and destruction of oxidation products of the angelicalactones can be schematically represented as shown in fig. 4.

t-Butyl hydrogen peroxide causes irreversible systolic standstill of the frog heart. Its effect is not at all modified by catalase, peroxidase, sodium pyruvate, or the substances which inhibit copper catalysis.

Considering the way in which their cardiac effect can be modified by catalase, peroxidase, sodium pyruvate, and the substances inhibiting metal catalysis, the oxidation products formed in the solution of the angelicalactones fall in between hydrogen peroxide and *t* butyl hydrogen peroxide.

Acknowledgments The author wishes to express his gratitude to Dr Otto Kraye for his help and advice during this work, and to Dr E. B. Astwood for the preparation of the milk and horseradish peroxidases.

REFERENCES

- (1) KRAYE, O., R. MENDEZ, E. MOISSY DE ESPANÉS AND R. P. LINSTAD, *THIS JOURNAL*, **74**, 372, 1942.
- (2) KRAYE, O., R. P. LINSTAD, AND D. TODD, *THIS JOURNAL*, **77**, 113, 1943.
- (3) KRAYE, O. *Proc Soc Exp Biol Med*, **53**, 51, 1943.
- (4) MITA, J. *Arch f exper Path u Pharmacol*, **104**, 276, 1924.
- (5) RICHTER, H., *Arch f exper Path u Pharmacol*, **194**, 362, 1940.
- (6) LYMAN, C. M., M. O. SCHULTZE AND C. G. KING, *J Biol Chem*, **116**, 757, 1937; DEXLER, A. O., AND R. C. DICKINSON *J Am Chem Soc*, **62**, 2165, 1940; STEINMAN, H. G., AND C. R. DAWSON *J Am Chem Soc*, **64**, 1212, 1942; GUZMAN BARRON, E. S., R. H. DeMeio, and F. Klemperer, *J Biol Chem*, **112**, 625, 1936.
- (7) MILAS, N. A., AND S. A. HARRIS, *J Am Chem Soc*, **60**, 2434, 1938.
- (8) ELLIOTT, K. A. C., *Biochem J*, **26**, 10, 1932.
- (9) KELLER, D., and T. MANN. *Proc Roy Soc London B*, **122**, 119, 1937.
- (10) SUMNER, J. B., AND G. F. SOMERS, *Chemistry and Methods of Enzymes* p. 171. New York Academic Press, 1943.
- (11) SCHALES, O., *Ber d Deut Chem Gesell*, **71**, 447, 1938.

PLASMA CONCENTRATIONS FOLLOWING THE ORAL ADMINISTRATION OF SINGLE DOSES OF THE PRINCIPAL ALKALOIDS OF CINCHONA BARK¹

EDWIN P. HIATT

Physiology Department of New York University College of Dentistry

Received for publication February 17, 1944

A study has been made of the plasma concentrations following the administration of an oral dose of each of the four principal cinchona alkaloids—quinine, quinidine, cinchonidine, and cinchonine—using normal human subjects. This study was undertaken for the purpose of determining the factors which influence the plasma concentrations of alkaloid achieved after administration of the mixed alkaloid preparations, Totaquina (U.S.P. XII). The plasma concentrations after oral doses of quinine have been described, but little is known about the plasma concentrations of the other alkaloids (1).

METHODS. The subjects were volunteer students from New York University College of Dentistry. The alkaloids were administered as the free bases, usually in gelatine capsules, about three hours after breakfast; but sometimes the powder was placed on the tongue and washed down with water. Blood samples were taken from the arm veins at one, two, three, five, and, in some cases, twenty-four hour intervals after the drug was administered. The subjects usually had lunch after the two-hour sampling. The blood samples were centrifuged immediately, and the separated plasma refrigerated until it could be analyzed. The estimation of alkaloid concentration was made by the colorimetric method devised by Brodie (2).

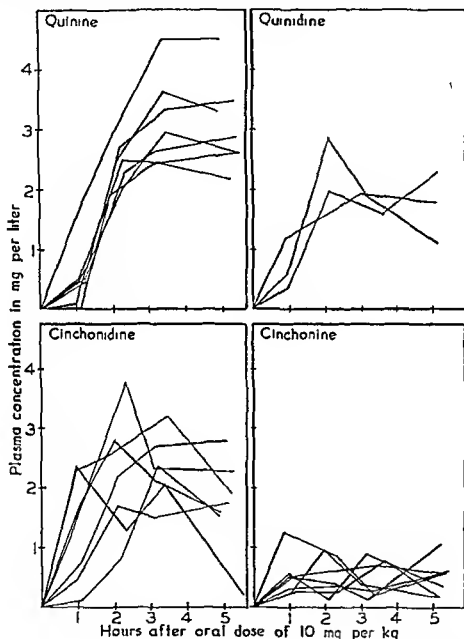
RESULTS. Typical experiments are summarized in the accompanying graphs. These represent experiments in which there was administered ten milligrams of the free alkaloid per kilogram of body weight. A number of additional experiments utilized smaller doses. However, the results were essentially the same except for a lower plasma level and need not be detailed here.

The data of figure 1 demonstrate a marked difference in the plasma concentration achieved after identical doses of the different alkaloids. The concentration after taking quinine is the highest, with quinidine and cinchonidine next, while cinchonine is very low. However, there is considerable variation in the curves obtained from the different individuals in the series. Because of this variation, a number of experiments were carried out in which several different alkaloids were given to the same individual. The experiments on four such individuals are presented graphically in figure 2. These fortify the data summarized in figure 1, in that the same differences are observed. Three of these subjects were observed following the administration of ten milligrams of Totaquina per kilogram of body

¹ The work described in this paper was in collaboration with other studies by The Research Service, Goldwater Memorial Hospital, done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and New York University.

weight. The concentration of alkaloid found after such a dose of Totaquina is of the expected order of magnitude, considering the composition of the mixture.²

Observations taken at 24 hours were of little help in the further examination of this aspect of the problem. In general, except for cinchonine, there was



- principal cinchona alkaloids in the plasma
an oral dose of 10 mgm. of the free base
alkaloid each curve represents a separate

remaining in the plasma about 0.2 to 0.8 milligrams per liter of plasma. The cinchonine had completely disappeared. There was no consistent difference

² This Totaquina (Lilly lot No. 43187) assayed by the second U.S.P. XII supplement: cinchonine, 26.46%, cinchonidine and quinine, 37.67%, quinidine, 9.47%, quinine, 11.34%. Total alkaloids, 72.99%.

among the remaining alkaloids. It is obvious that the further definition of the problem will require observations during the five to twenty-four hour interval.

Incidental to the running of the standard curves, observations were made in which the alkaloids were administered shortly after a meal. In all cases the

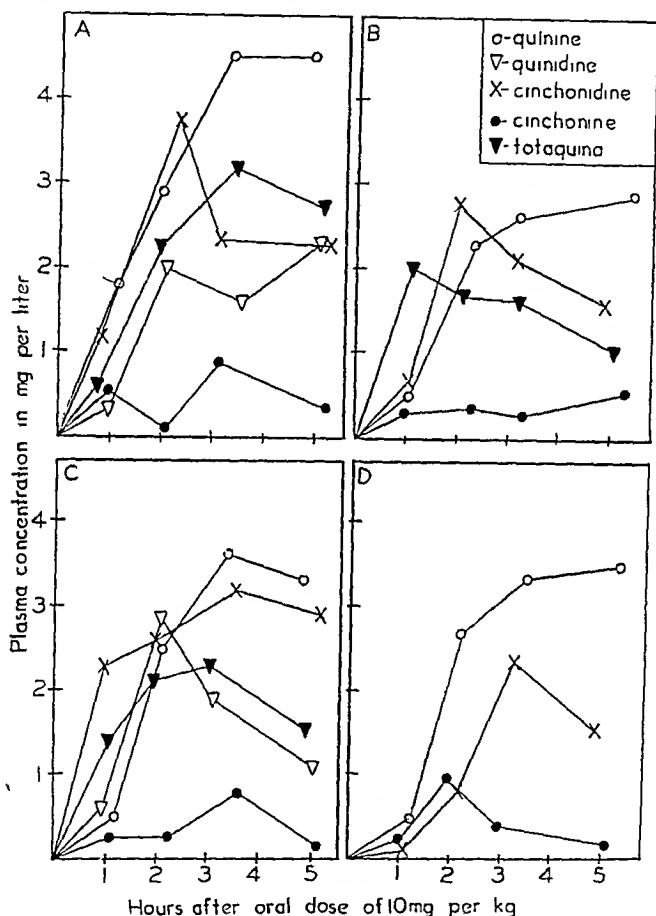


FIG. 2. The concentration of each of the four principal cinchona alkaloids in the plasma of normal men during the first five hours after an oral dose of 10 mgm. of the free base per kilogram of body weight. The curves in each block (A, B, C and D) represent data from a single individual.

plasma concentration during the next five hours was much lower than under the standard conditions. This would indicate that a major cause of variation in the plasma levels attained with equivalent doses of any one alkaloid is the amount of food in the stomach at the time of taking a dose.

No individuals manifested a definite idiosyncrasy to any of these alkaloids, although most reported mild symptoms of cinchonism with doses of ten milligrams per kilogram. These symptoms consisted of a feeling of fullness in the head, a heaviness of the eyelids, a dry mouth, and an exaggeration of the hand tremors. More than half of the subjects taking cinchonine had a period of nausea within an hour.

Discussion A most striking difference is apparent when the results on the optical isomers, cinchonidine and cinchonine, are compared. Such a difference in the case of two substances of similar molecular structure indicates that one or more of the cellular processes involved in the absorption, distribution, degradation, or excretion of these alkaloids (which together determine the plasma concentration) is capable of discriminating between these isomers.

It has been reported that all four alkaloids are about equally effective in the treatment of malaria when administered orally (1). Consequently if the anti-malarial activity of each alkaloid is proportional to its plasma concentration, then there are marked differences in their inherent anti-plasmodial activity. Furthermore, the therapeutic effectiveness of any mixture of the alkaloids, such as Totaquina, should not vary extensively with the proportions of the various constituents.

SUMMARY

1 Studies of the plasma concentrations, after taking oral doses of quinine, quinidine, cinchonidine, cinchonine, and Totaquina, were made using normal dental students as subjects.

2 With equivalent doses there are marked differences in the plasma concentrations reached after taking different cinchona alkaloids. Quinine gives the highest concentrations, cinchonidine and quinidine next, while cinchonine gives very low plasma concentration.

3 The plasma concentration after administering Totaquina is about what would be expected from the additive effect of the constituents.

4 The plasma concentration of any of the alkaloids is markedly lower if the dose is administered immediately after a meal.

REFERENCES

- (1) NELSON, E. E., *A Symposium on Human Malaria*. American Association for the Advancement of Sciences. No. 15, 1941.
- (2) BRODIE, B. B. personal communication.

STUDIES ON SHOCK INDUCED BY HEMORRHAGE

VII. THE DESTRUCTION OF COZYMASE AND ALLOXAZINE ADENINE DINUCLEOTIDE IN TISSUES DURING SHOCK¹

MARGARET E. GREIG

*From the Department of Pharmacology, Vanderbilt University School of Medicine,
Nashville, Tennessee*

Received for publication February 23, 1944

Abnormal increases in plasma of such substances as lactate, pyruvate, phosphate, potassium, creatine, thiamin, amino acids, and so forth, have been shown to occur in shock. The accumulation of intermediary metabolites in large amounts is, in part, a result of the anaerobic metabolism of the cell necessitated by the diminished oxygen supply to the tissues. Under anoxic conditions, since the cell becomes more permeable (1), substrates and other substances which normally are used by the cell diffuse into the plasma, where they are no longer able to be metabolized. The accumulation of intermediary metabolites as well as such substances as creatine, phosphate or thiamin is, however, also a result of a destruction of the enzyme systems themselves. Thus, it has been shown that one reason for the failure of pyruvate to be metabolized in shock induced by hemorrhage and in anoxic anoxia is that the specific coenzyme, coarboxylase, of pyruvic oxidase, becomes hydrolyzed by an enzyme present in the cell (2). The thiamin produced from this hydrolysis, due to its greater diffusibility, and to the increased permeability of the cell, diffuses into the blood stream where it is no longer utilizable as a coenzyme for intracellular metabolism (3). On the administration of large doses of thiamin to dogs in shock, some thiamin re-enters the cell and is synthesized to coarboxylase (2).

The effect of shock induced by hemorrhage on two other coenzymes essential for intracellular metabolism has now been investigated. Cozymase (coenzyme I or diphosphopyridine nucleotide), the nicotinamide containing coenzyme which is essential for the metabolism of a large number of substrates including lactate, malate, β -hydroxybutyrate, and diphosphoglyceraldehyde, and alloxazine adenine dinucleotide (flavine adenine dinucleotide), the riboflavin containing coenzyme, which is essential for the reoxidation of reduced cozymase as well as for the metabolism of many substances, among them amino acids, have been determined in muscle, liver and brain of control animals, and in animals before and after shock by hemorrhage.

GENERAL METHODS

Twenty-five dogs were used. Shock was induced in twenty, and five were used as controls.

¹ This work was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Vanderbilt University.

This paper was released for publication on February 12, 1944.

Anesthetic The dogs were anesthetized with pentobarbital sodium as described previously (4)

Production of shock Shock was induced by the method (4) already described which consisted of arterial bleedings at half hour intervals until the blood pressure remained below 60 mm. The amounts of blood removed were as follows: 2 bleedings of 1% of body weight, 2 of 0.5%, and all succeeding bleedings of 0.25%.

Tissues analyzed Analysis for cozymase and alloxazine adenine dinucleotide (A.A.D.) were done on samples of muscle, liver and brain. The first samples were removed from all dogs one hour after the animal had been anesthetized. In general the second samples were removed from the bled dog after he had been in shock for one hour, or after a shorter time if the animal were in such condition that he would not live for the full hour, and the third samples one hour after the second samples. In the control animals the second samples were removed three hours after the first and the third one hour later.

Muscle samples were taken with scissors from the deltoid muscles, liver by means of a cork borer, and cerebral cortex from holes trephined in the skull. A separate hole was made for each brain sample to avoid the possibility of obtaining damaged tissue, since damage has been found to accelerate destruction of cozymase.

The samples were weighted immediately after removal from the dog, plunged into boiling water, and heated at 100° for five minutes. The time required for these operations varied between 30 seconds and 1½ minutes. The tissues were snipped with scissors, homogenized, diluted to an appropriate volume and centrifuged.

PART I. COZYMASE

Method for determining cozymase Cozymase was determined by the method of Axelrod and Elvehjem (5) which was developed from von Euler's earlier method (6). The apozymase was prepared from Gerst's brewers' bottom yeast¹ in the following manner. The filtered yeast was spread out in a thin layer and dried under a fan at room temperature. Forty grams of dried yeast were stirred in 1600 cc. distilled water for 3½ to 4 hours. The suspended yeast was centrifuged, washed twice with distilled water and dried at room temperature under a fan. The dried apozymase when stored in a desiccator in the refrigerator remains active indefinitely. Fifty milligrams of most apozymase preparations was sufficient for one determination of cozymase, and produced volumes of CO₂ which were directly proportional to the amount of cozymase added up to 20 γ. The amounts of hexosediphosphate, glucose, buffer, magnesium and manganese were those recommended by Axelrod and Elvehjem (5). Nicotinamide, thiamin and riboflavin had no effect on the rate of fermentation. Standard curves were made for each set of determinations by adding 10 and 20 γ cozymase to the reaction mixtures.

Cozymase was prepared by the method of Williamson and Green (7). The purity of the preparation determined by the dithionite method and on the phosphorus content was found to be 70%.

RESULTS Table 1 shows the results of analyses for cozymase of tissues of control dogs. In most cases the tissues show either no change or an increase in cozymase. These are similar to the results obtained on co-carboxylase determinations in control animals. In two cases muscle shows some decrease. It should be pointed out that in obtaining samples some bleeding frequently occurred and there were occasionally transient decreases in blood pressure, which, however, never reached shock level. This might account for the changes occurring in muscle. In liver and brain no appreciable decreases occurred in the control animals.

In table 2 are given the results of cozymase determinations of dogs subjected

¹ Kindly supplied by the Gerst Brewing Company, Nashville, Tennessee

to hemorrhage. In contrast to the control animals these dogs showed frequent decreases in cozymase content of the tissues analyzed. For all animals subjected to hemorrhage the cozymase content of the second samples had decreased in 58% ($\frac{11}{19}$) of the cases for muscle, 42% ($\frac{8}{19}$) of the cases for liver and 60% ($\frac{12}{20}$) of the cases for brain. In some cases 3 samples were removed and no therapy was given to determine whether spontaneous increases in cozymase occurred. A spontaneous increase does not occur as evidenced by the fact that either no change or further decrease usually occurred between the second and third samples. In these dogs there was only one case, namely, muscle (dog 126) where an appreciable spontaneous increase in cozymase occurred. These decreases in

TABLE 1
Control dogs

DOG	TIME	COZYMASE IN γ /GRAM DRY TISSUE					
		Muscle	per cent change	Liver	Per cent change	Brain	Per cent change
98 (12.0 kg., ♂)	0			1855			
	1 hr.			1744	-6		
112 (9.0 kg., ♂)	0	1325		2190		1205	
	3 hr. 20 min.	1680	+27	2600	+19	1215	0
	4 hr. 15 min.	1345	-20	2500	0		
113 (7.5 kg., ♀)	0	1630		2430		1940	
	3 hr.	1770	+9	2680	+10		
	4 hr.	1790	0	2900	+8	2220	+14
133 (10.7 kg., ♀)	0	1660		968		948	
	3 hr.	1712	0	928	0		
	4 hr.	1670	0	1250	+35	1125	+19
140 (8.0 kg., ♀)	0	1470		2150		1180	
	3 hr.	970	-34	2680	+25	1336	+13
	4 hr.	1440	+48	2560	0	1640	+23

Values of less than 5% are considered as zero in all tables.

the third samples indicate that if shock is prolonged sufficiently all tissues eventually will show a destruction of cozymase. Brain seems to be the first tissue to be affected since it shows the most frequent decreases in the second sample; muscle is the next to be affected, and liver is the least affected. Liver may be the last to be affected since it is a storehouse for the vitamins and the destruction of cozymase may be prevented by the presence of excess nicotinamide, as found by Mann and Quastel (8).

Table 3 gives the results of experiments in which shock was induced by hemorrhage and vitamin therapy was given. In contrast to the dogs in table 2 the third samples removed after therapy usually show an increase in cozymase. The number of cases in which an increase occurred after administration of nicotinic

TABLE 2
Dogs subjected to hemorrhage—no therapy

DOG	PER CENT BLEEDING		COZYMASE IN γ /GRAM DRY TISSUE					
			Muscle	Per cent change	Liver	Per cent change	Brain	Per cent change
97 (16.2 kg, ♂)	3.0	Before shock	1775		3140		1785	
		After 60 min shock	1665	-6	2860	-9	1565	-12
99 (14.8 kg, ♀)	3.0	Before shock	1350				1180	
		After 30 min shock	1460	+8			070	-18
101 (0.4 kg, ♀)	4.4	Before shock	1580		3300		1660	
		After 25 min shock	1885	+19	2850	-14	1785	+7
105 (11.8 kg, ♀)	3.0	Before shock	1470		2910		1065	
		After 60 min shock	1055	-28	3720	+28	1270	+19
110 (9.4 kg, ♀)	4.75	Before shock	1945		1525		1590	
		After 20 min shock	1510	-22	1860	+22	1285	-10
119 (16.1 kg, ♀)	3.4	Before shock	1042		3160		1126	
		After 30 min shock	1073	0	1220	-65	832	-26
130 (9.0 kg, ♀)	3.82	Before shock	1140		2150		995	
		After 60 min shock	1250	+10	1700	-21	955	-4
131 (5.8 kg, ♀)	3.1	Before shock	896		652		772	
		After 60 min shock	804	-10	850	+30	662	-14
109 (11.0 kg, ♀)	?	Before shock	1174		1713		1220	
		After 60 min shock	1540	+31	1600	-7	880	-28
		No therapy	766	-50	1425	-11	842	-4
123 (9.0 kg ♀)	3.3	Before shock	1105		2400		1140	
		After 60 min shock	1440	+30	2365	0	838	-26
		No therapy	1070	-26	2240	-5	886	+5
126 (12.0 kg, ♀)	3.54	Before shock	1315		4060		890	
		After 60 min shock	954	-27	3630	-11	750	-16
		No therapy	1140	+20	2030	-44	524	-30
136 (8.45 kg ♀)	2.70	Before shock	992		1100		730	
		After 60 min shock	702	-20	1042	-5	395	-46
141 (11.0 kg, ♂)	3.5	Before shock	1520		2287		1010	
		After 60 min shock	1128	-26	1940	-15	809	-20
142 (11.0 kg, ♂)	4.45	Before shock			1800		695	
		After 60 min shock			3140	+75	820	+18

TABLE 3
Dogs subjected to hemorrhage followed by therapy

DOG	PER CENT BLEEDING		COZYMASE IN γ /GRAM DRY TISSUE					
			Muscle	Per cent change	Liver	Per cent change	Brain	Per cent change
100 (9.3 kg., ♀)	3.95	Before shock	1535		2840		1615	
		After 30 min. shock	1595	0	2750	-3	1450	-10
		Therapy (25 min. after TNRC)*			4140	+50		
103 (10.8 kg., ♂)	4.0	Before shock	1255		3120		980	
		After 30 min. shock	1195	-5	3670	+18	996	0
		Therapy (TRNC)	1220	+2	4430	+21	1130	+15
117 (7.7 kg., ♀)	3.75	Before shock	1210		1135		748	
		After 60 min. shock	803	-33	1410	+24	854	+14
		Therapy (N)	712	-11	2720	+93	1320	+55
128 (8.1 kg., ♀)	2.8	Before shock	868		1210		940	
		After 60 min. shock	1020	+18	1530	+26	824	-12
		Therapy (TRNC)	1075	+5	2240	+46	880	+7
132 (4.1 kg., ♂)	3.45	Before shock	1110		1330		804	
		After 60 min. shock	990	-11	1475	+11	818	0
		Therapy (RN)	1430	+45	930	-37	1135	+39
135 (7.0 kg., ♀)	4.0	Before shock	950		1038		740	
		After 60 min. shock	825	-13	1050	0	845	+14
		Therapy (RN)	1150	+39	1785	+70	758	-10

* T = thiamin, 5 mg./kg.; R = riboflavin, 5 mg./kg.; N = nicotinic acid, 10 mg./kg.; C = ascorbic acid, 5 mg./kg.

TABLE 4
Amount of bleeding required to produce shock correlated with the percentage destruction of cozymase
(Average bleeding of the group—3.58%)

AMOUNT OF BLEEDING IN PER CENT OF BODY WEIGHT	NO. OF CASES IN WHICH A DECREASE OF COZYMASE OCCURRED					
	Muscle		Liver		Brain	
Less than 3.58%.....	7/11	64%	5/10	50%	9/11	82%
More than 3.58%.....	4/7	57%	2/8	25%	2/8	25%
	NO. OF CASES IN WHICH AN INCREASE OF COZYMASE OCCURRED					
Less than 3.58%.....	3/11	27%	4/10	40%	1/11	9%
More than 3.58%.....	2/7	29%	4/8	50%	4/8	50%

acid, or nicotinic acid along with other vitamins was 60% ($\frac{3}{5}$) for muscle, 83% ($\frac{5}{6}$) or liver and 80% ($\frac{4}{5}$) for brain. The amount of cozymase synthesized frequently exceeded that present in the animal at the start of the experiment.

In table 4 the amount of bleeding necessary for the induction of shock is correlated with the number of cases in which destruction of cozymase occurred. It will be seen that the group of dogs which went into shock with less than the average amount of bleeding contained a larger number of cases in which destruction of cozymase occurred. This is particularly true of brain where 82% of the low bleeding group showed cozymase destruction, while only 25% of the high bleeding group showed a decrease. On the other hand the tendency for some synthesis of cozymase to occur was greater in the group which required a large amount of bleeding to produce shock. In brain 9% of the low bleeding group showed synthesis while 50% of the high bleeding group showed an increase of cozymase on bleeding.

DISCUSSION The disappearance of cozymase especially on destruction of the cell structure has been observed by von Euler and co workers (9, 10, 11). In the experiments here reported destruction of cozymase was found to occur most frequently in brain, less in muscle and least in liver. The findings of Das and von Euler (12), that the reduced form of cozymase is more easily hydrolyzed by tissues than is the oxidized form, may explain the destruction of cozymase which has been found to occur in shock, since the reduced form would probably accumulate as the tissues become anoxic.

Brain appears to be the first tissue to become affected by hemorrhage since it shows decreases in cozymase most frequently. However, if shock is sufficiently prolonged other tissues eventually show cozymase destruction since the cozymase content of the third samples of animals which did not receive therapy showed further decreases.

Under the conditions of our experiments administration of nicotinic acid is essential for a resynthesis of cozymase since in animals subjected to a similar degree of shock without therapy there is rarely a spontaneous resynthesis of cozymase. This would indicate that the administration of nicotinic acid to animals in shock might prove beneficial in restoring the metabolism to normal.

The finding that there is an inverse correlation between the amount of bleeding necessary to produce shock and the destruction of cozymase is in agreement with the earlier work on shock. Govier (3) found that the incidence of intestinal hemorrhage after bleeding was much higher in the group of animals which went into shock with a small amount of bleeding than in that group which required a large amount of bleeding to produce persistent hypotension. That the breakdown of cozymase might be associated with intestinal bleeding is suggested by the work of Calder and Kerby (13) who found that nicotinic acid was beneficial in various hemorrhagic syndromes.

PART II ALLOXAZINE ADENINE DINUCLEOTIDE

Method for determining Alloxazine Adenine Dinucleotide (A.A.D.) A.A.D. was determined by the method of Warburg and Christian (14). The apoenzyme of d-aminooxidase was prepared from either dog or sheep kidneys¹ and A.A.D. from either Fleischmann's bakers yeast or dog liver. The purity of the A.A.D. was determined by making use of the data of Warburg and Christian who used A.A.D. of 100% purity and found

¹ Kindly supplied by the Neuhoft Packing Company, Nashville, Tennessee.

that the reaction velocity was one-half its maximum when the concentration of A.A.D. was 0.196 γ per cc. The purity of preparations varied between .07 and .26. Solutions were made which contained 1 γ pure substance per cc. and were stored in the frozen state. A standard curve was made for each set of analyses and results are expressed as γ per gram dry tissue.

RESULTS. The results of determinations of A.A.D. on muscle, liver and brain of control dogs are given in table 5. It will be seen that in most cases an increase in A.A.D. occurred. In one case for muscle and one for liver there was a decrease. As already stated there was always some bleeding and probably some degree of shock was produced in obtaining samples, which might account for such de-

TABLE 5
Control dogs

DOG	TIME	ALLOXAZINE ADENINE DINUCLEOTIDE IN γ /GRAM DRY TISSUE					
		Muscle	Per cent change	Liver	Per cent change	Brain	Per cent change
98 (12.0 kg., ♂)	0			201			
	1			276	+37		
112 (9.0 kg., ♂)	0	107		276		65	
	3	115	+7	238	-14	64	0
	4	131	+14	234	0	66	0
133 (10.7 kg., ♀)	0	119		604		82	
	3	128	+8	600	0	96	+17
	4	132	+31	644	+7	115	+20
140 (8.0 kg., ♀)	0	125		504		60	
	3	104	-17	625	+24	63	+5
	4	121	+16	598	0	70	+11

creases in coenzyme in the control animal. In no case was there a decrease of A.A.D. in brain.

Table 6 gives results of A.A.D. determinations on dogs before and after hemorrhage. It will be seen that appreciable decreases in the A.A.D. content of the tissues examined occur when the dog is bled. The number of cases in which a decrease occurs is 62% ($\frac{8}{13}$) for brain, 46% ($\frac{6}{13}$) for muscle and 8% ($\frac{1}{12}$) for liver. Administration of riboflavin can result in a resynthesis of A.A.D., although the effects are not so marked as in the case of cozymase.

DISCUSSION. The changes in the A.A.D. concentrations which occur with the onset of shock are similar to those found for cozymase. A destruction of A.A.D. occurs most frequently in brain and least frequently in liver when the animal is bled. The results are variable as one would expect since it is possible that a certain number of the animals were not in shock. However, the bled dogs as a group do behave differently from the control group. On the administration of riboflavin to dogs in shock a resynthesis of A.A.D. can occur quite rapidly in

TABLE 6
Dogs subjected to hemorrhage

Dog	PER CENT BLEEDING		ALLOXAZINE ADFNINE DINUCLEOTIDE IN γ /GRAM DRY TISSUE					
			Muscle	Per cent change	Liver	Per cent change	Brain	Per cent change
97 (16.2 kg, ♂)	3.3	Before shock	103		455		70	
		After 60 min shock	58	-44	436	-4	86	+23
99 (14.8 kg, ♀)	3.0	Before shock	74				53	
		After 30 min shock	08	+32			40	-24
100 (9.3 kg, ♀)	3.95	Before shock	90		432		61	
		After 30 min shock	68	-24	438	0	58	-6
101 (9.4 kg, ♀)	4.4	Before shock	96		346		57	
		After 25 min shock	01	-5	506	+46	58	0
102 (13.0 kg, ♂)	3.9	Before shock	85		425		52	
		After 60 min shock	113	+33	410	-3	33	-37
110 (9.4 kg, ♀)	4.75	Before shock	122		468		83	
		After 20 min shock	115	-6	454	0	77	-7
126 (12.0 kg, ♀)	3.54	Before shock	120		704		63	
		After 60 min shock	100	-17	750	+7	66	+5
		Therapy (RNC)	120	+20	715	-4	53	-21
128 (8.1 kg, ♀)	2.8	Before shock	147		432		92	
		After 60 min shock	165	+12	398	-8	82	-11
		Therapy (TRN)	191	+16	443	+11	76	-7
130 (9.0 kg, ♀)	3.82	Before shock	81		708		85	
		After 60 min shock	125	+54	780	+10	72	-15
131 (5.8 kg, ♀)	3.1	Before shock	66		455		64	
		After 60 min shock	76	+15	500	+10	56	-13
132 (4.1 kg, ♂)	3.45	Before shock	92		471		28	
		After 60 min shock	62	-32	472	0	41	+46
		Therapy (RN)	73	+18	518	+9	50	+22
135 (7.0 kg, ♀)	4.0	Before shock	77		358		53	
		After 60 min shock	78	0	342	-4	63	+19
		Therapy (RN)	73	-6	454	+33	61	0
141 (11.0 kg, ♂)	3.5	Before shock	127		710		58	
		After 60 min shock	133	+5	758	+7	49	-16

liver and muscle. It is possible that a longer time than our experimental period is required for synthesis to occur in brain. Ochoa and Rossiter (15) found that on the administration of riboflavin to deficient animals a synthesis occurred in

one-half hour in liver but that a longer time was required before a synthesis could be observed in heart.

The destruction of A.A.D. in shock may be one factor leading to the accumulation of amino acids as found by Lurje (16) and Engel et al. (17).

It has now been shown that destruction of three coenzymes, namely, co-carboxylase, cozymase, and alloxazine adenine dinucleotide, may occur in shock.

That the onset of shock is accompanied by the breakdown of coenzymes is shown by the following facts:

1. Resistance of dogs to shock was found to be significantly greater in those animals having high plasma thiamin levels than in those showing low plasma thiamin values (3).

2. Animals which were susceptible to shock showed a diffusion into the plasma of large amounts of thiamin, indicating a breakdown of tissue co-carboxylase (3).

3. Co-carboxylase was found to decrease in muscle, liver and duodenum in animals subjected to hemorrhage and to anoxic anoxia (2).

4. Some degree of correlation was found between the amount of bleeding necessary for the onset of shock and the degree of destruction of co-carboxylase (2).

5. Cozymase and A.A.D. were found to decrease frequently in brain, muscle and liver in shock.

6. Dogs requiring more than average amounts of bleeding to go into shock showed less destruction of tissue cozymase than did dogs which went into shock with small amounts of bleeding.

From these results it would seem obvious that it is necessary to keep the coenzymes intact. For the synthesis of these coenzymes in animals in shock, under our experimental conditions, vitamin therapy has been found to be essential.

SUMMARY

1. Changes in cozymase and alloxazine adenine dinucleotide are found to occur in muscle, liver and brain when the animal is subjected to hemorrhage.

2. Brain is apparently the first tissue to show the effect of hemorrhage since decreases in cozymase and alloxazine adenine dinucleotide are most frequent in this tissue.

3. Animals requiring large amounts of bleeding to produce shock showed less destruction of cozymase than did animals which went into shock with small amounts of bleeding.

4. Administration of nicotinic acid and riboflavin may result in a resynthesis of the respective coenzymes.

REFERENCES

- (1) DIXON AND HOLMES, *Nature*, **135**, 995, 1935.
- (2) GREIG AND GOVIER, *THIS JOURNAL*, **79**, 169, 1943.
- (3) GOVIER, *THIS JOURNAL*, **77**, 40, 1943.
- (4) GOVIER AND GREER, *THIS JOURNAL*, **72**, 321, 1941.
- (5) AXELROD AND ELVEHJEM, *J. B. C.*, **131**, 77, 1939.

- (6) VON EULER AND MYRBACK, Z physiol chem, 131, 179, 1923
- (7) WILLIAMSON AND GREEN, J B C, 135, 345 1910
- (8) MANN AND QUASTEL, Biochem J, 35, 502 1941
- (9) VON EULER, MYRBACK, BRUNIUS, Z physiol chem, 177, 237, 183, 60, 1929
- (10) VON EULER AND GUNTHER Z physiol chem, 243, 1, 1936
- (11) VON EULER, HEIWINKEL, SCHLENK, Z physiol chem, 247, IV, 1937
- (12) DAS AND VON EULER, Nature, 141, 601, 1938
- (13) CALOER AND KERRY, Am J Med Sci, 200, 500, 1940
- (14) WARBURG AND CHRISTIAN, Biochem Z, 298, 150, 1938
- (15) OCHOA AND ROSSITER, Biochem J, 33, 2008, 1939
- (16) LURJE, Am J Surg, 32, 313, 1936
- (17) ENGEL, WINTON AND LONG, J Exper Med, 77, 397, 1913

THE ANTISPASMODIC ACTIVITY OF SOME 4-MORPHOLINEALKYL ESTERS*

I. TOXICITY, ISOLATED SMOOTH-MUSCLE EFFECTS AND SPASMOLYTIC ACTIVITY ON THE ILEUM OF ANESTHETIZED DOGS

HAROLD F. CHASE, ARNOLD J. LEHMAN AND FREDRICK F. YONKMAN

Department of Pharmacology and Therapeutics, Wayne University College of Medicine, Detroit, Michigan

Received for publication February 28, 1944

Innumerable attempts have been made to find a readily synthesized compound with the antispasmodic properties of the naturally occurring alkaloids, atropine or papaverine, without the distressing side effects of atropine or the legal and economic limitations associated with the use of papaverine. Cheney and Bywater have synthesized a group of 4-Morpholinealkyl esters and amides (1) of which the esters were examined by Rowe (2) for antispasmodic properties and toxicity. Of the thirty-two compounds studied by Rowe, eight having the most favorable toxicities and the greatest spasmolytic potencies were subjected to more extensive investigation. This paper concerns itself with these eight agents and is an evaluation of their toxicity, isolated smooth muscle effects and spasmolytic activity on the ileum of anesthetized dogs. Experiments are now in progress to determine the depressant properties on intestinal motility of unanesthetized dogs with various types of fistulae, the results of which will be reported in a later communication. A preliminary report of the bronchodilating effects of certain of these morpholine compounds has been published (3).

For convenience this group of compounds has been designated as series 'S' whose individual chemical names are as follows:

S-5 β -4-Morpholineethyl diphenylacetate HCl

S-9 γ -Morpholinepropyl diphenylacetate HCl

S-14 β -4-Morpholineethyl diphenylchloroacetate HCl

S-19 β -(β -4-Morpholineethoxy)-ethyl diphenylacetate HCl

S-28 β , β -Dimethyl- γ -4-morpholinepropyl diphenylacetate HCl

S-29 ω -4-Morpholinehexyl diphenylacetate HCl

S-35 β -4-Morpholineethyl phenylcyclohexaneacetate HCl

S-37 β , β -Dimethyl- γ -4-morpholinepropyl phenylcyclohexaneacetate HCl

Hereafter these substances will be referred to by their group designation (i.e. S-5) for the sake of brevity.

TOXICITY. The toxicity of the 'S' compounds has been studied in white mice following intraperitoneal injection and in white rats following intravenous administration. The intraperitoneal injections were given as one per cent solutions in physiological saline in doses calculated in milligrams per kilogram. Mice

* This presentation represents part of the antispasmodic program which is supported by a research grant established in this department by Parke Davis and Company.

weighing eighteen to twenty five grams were employed and then observed for twenty four hours for fatal outcome. The intravenous doses were given as one per cent solutions into the tail vein of white rats weighing two hundred to two hundred and fifty grams. Injections were given slowly enough to allow the rats to accommodate the volume of fluid and these animals were likewise observed for twenty four hours. The number of animals used in these determinations totaled six hundred white mice and three hundred and thirty five white rats.

The results are compiled in table 1 which presents values for the maximum tolerated dose, the lethal dose in fifty per cent of the animals (LD_{50}) as determined by the method of Reed and Muench (4) and the lethal dose one hundred per cent (LD_{100}) for both intraperitoneal and intravenous administration. Diethylaminoethyl diphenylacetate HCl (Trasentin) is included for the purpose of comparison.

TABLE 1
Toxicity of the morpholine derivatives in rats and mice

DRUG	MICE—INTRAPERITONEAL			RATS—INTRAVENOUS		
	Maximum tolerated dose	LD_{50}	LD_{100}	Maximum tolerated dose	LD_{50}	LD_{100}
	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
S 5	200.0	576.9	800.0	25.0	41.2	50.0
S 9	250.0	315.0	400.0	15.0	19.8	25.0
S 14	200.0	234.0	400.0	50.0	53.5	60.0
S 19	100.0	209.0	500.0	12.5	15.7	25.0
S-28	2000.0			50.0	62.8	100.0
S 20	300.0	135.5	500.0	12.5	20.6	30.0
S-35	450.0	593.7	750.0	25.0	49.0	65.0
S 37	1500.0			50.0	63.0	75.0
Trasentin	100.0	185.0	300.0	10.0	17.3	25.0

The derivatives with the lowest toxicity were S-28 and S-37. Using one per cent solutions it was physically impossible to inject into the peritoneal cavities of mice enough of either compound to cause death of any animals even when doses as high as 1500 and 2000 mg/kg were given. These same substances were also least toxic intravenously since the LD_{50} doses were 62.8 and 63.0 mg/kg respectively. The extremely low toxicity after intraperitoneal injection may be explained by the fact that both of these compounds were difficultly soluble in physiologic fluids, therefore precipitation with subsequent slow absorption may have occurred in the peritoneal cavity.

The next most innocuous of these morpholine congeners at the LD_{50} level were S-35 and S-5. However when the maximum tolerated doses (MTD) of these two are considered their desirability is lessened. The differences between LD_{50} and MTD for all compounds averaged 135 mg/kg intraperitoneally and 8.5 mg/kg intravenously. The LD_{50} — MTD difference for S-5 intraperitoneally was three times as great as the average and for S-35 intravenously was four times the average. Therefore these agents were not as innocuous as would appear at first glance.

S-14 is an exception to the close parallelism which prevails in a comparison of the intraperitoneal and intravenous LD_{50} 's. It has a low toxicity intravenously, but the converse is true by the other route of administration.

S-19 and S-9 are the most toxic of these compounds, while S-29 is intermediate.

ANTISPASMODIC ACTIVITY ON ISOLATED TISSUE. *Intestinal.* Rabbit gut was subjected to trial both by the Magnus method (5, 6) employing isolated smooth muscle segments and by the Trendelenberg method (7) using excised loops. Oxygenated Sollman-Rademacher's solution without glucose was the medium used in both techniques, and drugs were added directly to the bath. Not only were the direct effects of the drugs observed in the absence of previously induced stimulation, but their abilities to antagonize a typical musculo-stimulant drug

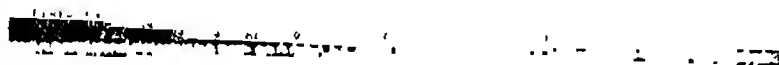


FIG. 1. ACTION OF S-29 ON TRENDLENBERG LOOP OF RABBIT ILEUM PREVIOUSLY STIMULATED BY BARIUM CHLORIDE

Effects shown and longitudinal increased tonus, failed to relax this spasm whereas in 1:500,000 concentration it was effective in lowering longitudinal tonus and in restoring perfusion output, but did not affect circular muscle nor restore the rhythm of longitudinal muscle.

such as barium chloride in a strength of 1:5000 and a typical neurotropic drug like pilocarpine at 1:100,000 concentration were also quantitated.

There was no clear-cut evidence of differential action between musculotropic and neurotropic depression of intestinal smooth muscle by any of the 'S' compounds, since their effective doses in each of the tests applied varied only slightly. The few instances in which suggestive variations did occur were specifically: S-14, which responded by antagonizing pilocarpine with a weaker concentration in both techniques, S-19 on the Trendelenberg loop, on which it was especially active in relaxing barium spasm, and S-28, which was quite irregular in its responses. Because of the general uniformity of response, we have presented the results as effective dosage ranges for each derivative.

None of the members of this series approached very closely the consistent

spasmolytic properties of S 29 which was effective in doses ranging from 1 250,000 to 1 300,000 by the Magnus technique and from 1 125 000 to 1 500,000 by the Trendelenberg method. S 19 was two thirds as active and S 14, S 28 and S 35 were each about one third as active as S 29. Because of the tendency of S 28 to precipitate in Sollman Rademaker's solution, quantitation of its activity by these techniques was judged to be not entirely indicative of its power as a smooth muscle relaxant.

Uterine. In a similar manner the antispasmodic abilities of these new substances were tested on isolated strips of rabbit uterine smooth muscle, both directly and in antagonism of the stimulation resulting from previous administration of barium chloride 1 5000 and histamine 1 100 000 concentrations. The concentrations necessary to depress uterine muscle were greater on the average than those producing similar results on the intestinal muscle. Here again S 29 in effective dilutions of 1 150 000 for each situation studied, whether unopposed

TABLE 2

Effective spasmolytic doses of the morpholine compounds on excised tissues

DRUG	EXCISED RABBIT INTESTINE		EXCISED RABBIT UTERUS 3 CM'S METHOD
	Magnus Strip Method	Trendelenberg Loop Method	
S 5	1 12 500-1 25 000	1 50 000-1 100 000	1 5 000-1 10 000
S 9	1 12 500-1 25 000	1 50 000-1 75 000	1 7 500-1 25 000
S 14	1 10 000-1 50 000	1 100 000-1 300 000	1 2 500
S 19	1 250 000-1 300 000	1 25 000-1 250 000	1 50 000-1 100 000
S 28	1 50 000-1 150 000	1 43 000-1 125 000	1 12 500-1 50 000
S 29	1 250 000-1 300 000	1 250 000-1 500 000	1 150 000
S 35	1 100 000-1 130 000	1 50 000-1 75 000	1 50 000-1 60 000
S-37	1 50 000-1 75 000	1 7 500-1 10 000	1 7 500-1 15 000
Trasentin	1 100 000-1 300 000	1 62 500-1 500 000	1 25 000-1 50 000

or antagonized, was by far the most consistently depressant congenit. S 19 and S 35 averaged respectively one half and one fourth of the activity of S 29 and showed no essential differences in response to antagonistic agents. S 28 gave promise of activity directly, but failed to bear out this promise in competition with stimulant drugs.

ANTISPASMODIC ACTION ON ILEUM OF ANESTHETIZED DOGS. Jackson's internal organ apparatus (8) was attached to the ileum of dogs anesthetized with sodium pentobarbital. Urethane anesthesia was tried and discarded since it was not as uniform and controllable as that produced by sodium pentobarbital, neither did the barbiturate seem to interfere with motility in any noticeable degree. Two drugs, S 5 and S 9 were eliminated in the preliminary studies as the least effective spasmolytic agents and were not used in this phase of the project. The other S compounds were injected intravenously as one per cent solutions in physiologic saline in doses of 1.0 and 5.0 mg./kg. Three to five experiments were performed in testing each of the compounds employed at each of the above doses, both on dogs whose ilia were normal or unstimulated and on animals whose intestinal

motility had been activated by subcutaneous injection of two international units of posterior pituitary solution per kilogram. The total number of injections of each of the drugs ranged from thirteen to fifteen.

TABLE 3

Effects of morpholine compounds on intestinal motility and arterial pressure in anesthetized dogs

DRUG	TOTAL NUMBER INJECTIONS	SPASMOLYTIC EFFECTS ON ILEUM					FALL ARTERIAL PRESSURE, 5.0 MG./KG. DOSE
		1 0 mg./kg.		5 0 mg./kg.		Order of effective- ness	
		Unstimulated	Stimulated	Unstimulated	Stimulated		
S-14	13	Moderate	Moderate	Marked	Moderate	3	<i>per cent</i> 5
S-19	15	Moderate	Slight	Moderate	Moderate	4	20
S-28	15	None	None	Slight	Slight	6	5
S-29	15	Moderate	Marked	Marked	Marked	1	15
S-35	14	Slight	None	Moderate	Moderate	5	17
Trasentin	14	Marked	Moderate	Marked	Marked	1	19

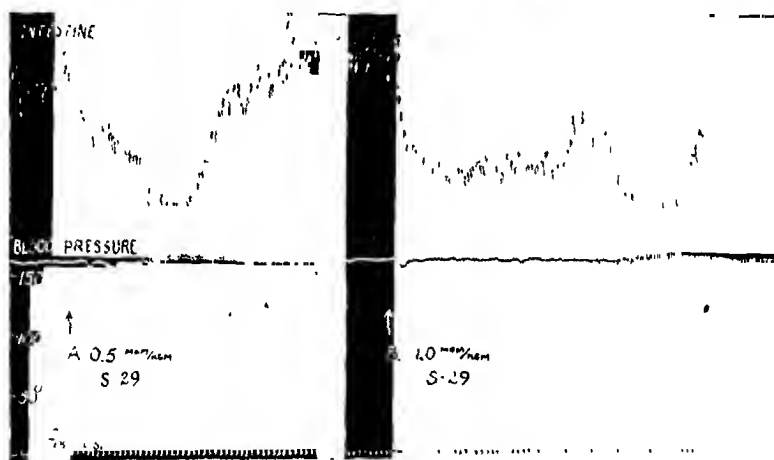


FIG. II. DOG PENTOBARBITAL ANESTHESIA

The effects of S-29 in doses of 0.5 at A and 1.0 mg./kg. at B on ilial motility and arterial pressure. Note the graded response of the intestine to increased dosage and the very slight alteration of arterial blood pressure accompanying the injection of S-29.

The effect of intravenous injection of the morpholine compounds on arterial pressure was observed simultaneously with their depressant properties in those dogs not prepared with posterior pituitary solution.

The relative order of activity of the various derivatives was essentially the same following doses of 5.0 mg./kg. and 1.0 mg./kg. differing mainly in the degree of response. The essential features of the experimental results are shown in table 3.

S 29 was most outstanding, showing at 50 mg/kg in the unstimulated gut a reduction of peristalsis to almost complete inactivity for an average duration of 21.5 minutes. Fig. II presents a typical tracing showing the action of S 29.

The next most potent member was S 14 which showed depressant action which was marked but not equal in intensity or duration to S 29. The other derivatives were much weaker. Because of its extremely low toxicity S 28 was given in higher doses, sometimes as a 2.0% solution, but even with 20.0 mg/kg its activity was scarcely as great as the least effective of the other agents with 50 mg/kg.

When tested in dogs whose intestines had been activated by pituitrin, S 29 was even more outstanding in comparison with the other agents. At 50 mg/kg it reduced rhythm and tonus completely for a duration of nine minutes.

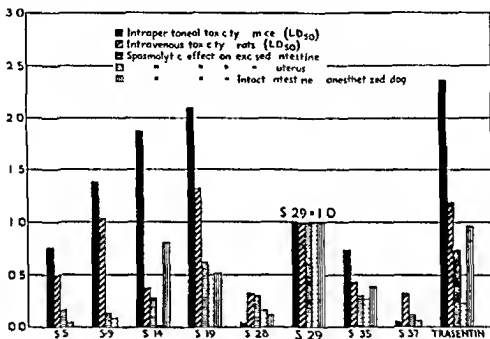


FIG. III. RATIOS OF TOXICITY AND SPASMOLYTIC ACTIVITY OF THE MORPHOLINE COMPOUNDS

Under these circumstances also, S 14 was the next most potent representative of the group. The other compounds were not sufficiently effective to be worthy of discussion.

The chief circulatory effect of intravenous injection of any of the morpholine compounds was a transient drop in arterial pressure proportionate to the dose given. At 10 mg/kg the fall was a negligible 2 to 4% while at 50 mg/kg the range was from 5 to 20% averaging 12%.

DISCUSSION. An appraisal of all antispasmodics studied in this investigation gives a clear impression of the outstanding merit of S 29 from the point of view of moderate toxicity combined with superior activity. Fig. III presents a graphic review of the data on toxicity and spasmolytic properties of all members of the series, employing S 29 as unity. The ratios in the graph are based upon the LD₅₀, the average effective doses, or the responses to standardized doses of S 29. This manner of presentation serves to re-emphasize the statements re-

garding the superiority of this agent as an antispasmodic. Particular notation should be made of its outstanding performance as a uterine depressant.

The graph demonstrates that S-19 was also prominent for its uterine action and had moderate potency on the isolated and intact intestine. However its toxicity was relatively high. The varying responses of S-14 are likewise illustrated. It was the most toxic intraperitoneally, but of low toxicity intravenously. It was quite effective on isolated intestinal tissue, was second to S-29 throughout the anesthetized dog gut experiments, but was almost inactive on excised uterus. S-35 combined low toxicity with moderate spasmolytic power.

Rowe (2), in his preliminary studies on this group of 4-morpholine alkyl esters, reported that S-28 was twenty-five per cent more active than S-29 on Magnus strips and exhibited very low toxicity. We were able to confirm the low toxicity, but we were unable to demonstrate such high potency as an antispasmodic. The sample which we used was very difficultly soluble and tended to precipitate in physiologic solutions. Despite this precipitation we have seen suggestive evidence that it has considerable activity, particularly against non-stimulated intestinal and uterine strips. In the light of Rowe's findings and our own we believe that this congener merits further consideration. It is now being investigated further in this laboratory by enteral administration to unanesthetized animals, the results of which experimentation will be reported in a later communication.

Diethylaminoethyl diphenylacetate HCl (Trasentin) a synthetic compound of a chemical nature similar to the morpholine compounds, and a product which is commercially available at the present time as an antispasmodic, was included in these studies for the purpose of comparison. In our studies its toxicity was greater than that of S-29; namely, 2.35 times as toxic intraperitoneally in mice and 1.19 times as toxic intravenously in rats (Fig. III). On the intestine it was less potent when applied to the unstimulated excised gut but about equipotent in antagonism of stimulating drugs. Its average activity upon this tissue was 0.74 times that of S-29. In direct action and in antispasmodic (antagonistic) action upon isolated uterine strips S-29 was five times as strong as Trasentin. When administered intravenously to anesthetized dogs Trasentin showed a slightly more intense and more prolonged action on the unstimulated gut, but following pituitrin stimulation, the situation was reversed (table 3). S-29, the most effective of the morpholine compounds included in the present study, is much less toxic and is of equal or greater potency than Trasentin, especially on uterine smooth muscle. It would seem, therefore, that S-29 would merit clinical trial as an antispasmodic.

The data from these experiments, particularly those on isolated intestinal tissue, would tend to suggest a muscular rather than an anticholinergic (neural) seat of antispasmodic action since the effective dosages are so nearly alike, for direct depression, for musculostimulant and for neuro-stimulant antagonism. There is no convincing differentiation.

SUMMARY

1 Eight morpholine alkyl esters have been examined for toxicity, for anti spasmodic activity on excised intestine and uterus and on the ileum of anesthetized dogs

2 The evidence presented suggests that these compounds act as spasmolytic agents by direct depression of smooth muscle

3 S 29, ω 4 Morpholinehexyl diphenylacetate HCl, is the outstanding member of the series from the point of view of moderate toxicity combined with superior spasmolytic activity on the intestine and especially on the uterus. On the basis of this investigation it would seem to merit clinical trial

BIBLIOGRAPHY

- (1) CHENEY AND BYWATER J Am Chem Soc 64, 970-973 1942
- (2) ROWE, J Am Pharmaceut Assoc 31, 57-59 1942
- (3) CHASE LEHMAN AND LONKMAN Federation Proceedings 2, 76 1943
- (4) REED AND MUENCH Am J Hygiene 27, 439 1938
- (5) MAGNUS Arch ges Physiol 102, 123 1904
- (6) MAGNUS Ibid 103, 515 1904
- (7) TRENDLENBERG Arch f exp Path Pharm 81, 55 1917
- (8) JACKSON Experimental Pharmacology and Materia Medica C V Mosby & Son figs 145 and 146

THE COMPARATIVE ANOXEMIC EFFECTS FROM CARBON MONOXIDE HEMOGLOBIN AND METHEMOGLOBIN

DAVID LESTER AND LEON A. GREENBERG

From the Laboratory of Applied Physiology, Yale University, New Haven, Conn.

Received for publication, March 8, 1944

Darling and Roughton (1) have recently shown that the formation of methemoglobin, like that of carbon monoxide hemoglobin, causes a shift to the left in the oxygen dissociation curve of the blood. In consequence, methemoglobin, like carbon monoxide hemoglobin, should have an adverse effect upon the respiratory functions of the blood beyond that of the direct inactivation of a portion of the hemoglobin but this effect should be less than for carbon monoxide since the shift to the left is less. Darling and Roughton made no study on the living animal to determine the comparative impairments caused by methemoglobin and carbon monoxide hemoglobin. They accept the statements in the literature (2), based on no critical evaluation, that the symptoms of anoxemia from methemoglobin formation are very similar to those produced by an equal formation of carbon monoxide hemoglobin. From this assumption, which is not supported by more recent work of Vandenbelt, Pfeiffer, Kaiser and Sibert (3), they are led to draw rather general conclusions as to the clinical significance of methemoglobinemia produced by drugs. They say: "Although 20 to 30 per cent methemoglobin should be tolerated well by normal individuals, just as is 20 to 30 per cent CO hemoglobin, more than that would be likely to lead to serious tissue anoxemia, quite independently of any direct toxic action of the methemoglobin-producing agent on the tissues." They point out further that the danger point might well be reached at lower concentrations of methemoglobin in "ill subjects." "Ill subject," as used, is too general a term; for anoxemia from the therapeutic use of methemoglobin-producing drugs, illness would have to be limited to ailments in which there is already a burden upon the transportation of oxygen. The emphasis, and in quantitative terms, that they put upon the clinical dangers of methemoglobinemia would, in the wide use of methemoglobin-forming drugs, particularly of the sulfonamide group, seem to require more proof than is given of the comparable effects of carbon monoxide hemoglobin and methemoglobin.

Our attention was directed to this matter by two observations: First, in laboratory experiments we have frequently observed animals with methemoglobinemia to the extent of 80 or even 85 per cent which were not unconscious, whereas 60 to 70 per cent carbon monoxide hemoglobinemia is generally believed to cause unconsciousness and 70 to 80 per cent death (4, 5). Vandenbelt, et al. (3) report recovery in dogs with 82 to 87 per cent methemoglobin; and death at 94 to 95 per cent methemoglobin after administration of p-aminopropiophenone. And second, the formation of methemoglobin from such aniline derivatives as we have studied (but not that from nitrites), unlike carbon monoxide hemoglobinemia for any total amount of carbon monoxide absorbed, is influenced by the

total hemoglobin present and exhibits a ceiling value which varies with the different drugs, and which is reached at a level below that of fatal asphyxiation (6, 7)

Darling and Roughton point out that in the past it has been thought that the severity of the effects seen during methemoglobinemia, as compared to a similar loss of hemoglobin from anemia, was due to the direct effects of the methemoglobin forming agent. Their demonstration of a shift in the dissociation curve has apparently been taken by them as a full explanation for the differences in severity. While undoubtedly this shift plays some part, we believe that in many instances of experimentally induced methemoglobinemia an even greater part is played by the toxic action of the agent used or by the exacerbation of the anoxemia of the brain by a fall in blood pressure as from nitrite. We believe further, that in any ordinary therapeutic use of the common drugs that produce methemoglobin, the asphyxia is of minor importance. We have shown that with nitrite as ordinarily administered in experimental studies in a single large dose, a considerable amount of nitrite remains in the blood even at the point of maximum formation of methemoglobin (8). With such methemoglobin forming substances as *p*-aminophenol, the conversion may cease at a percentage which does not cause severe asphyxia, but if the dose is sufficiently large the animals will die without further formation of methemoglobin (7).

The present study was made in an attempt to evaluate the comparative anoxemic effects on the living animal resulting from the formation of carbon monoxide hemoglobin and methemoglobin. Cats were used mainly as the experimental animals.

THE OXYGEN DISSOCIATION CURVE IN THE PRESENCE OF METHEMOGLOBIN

For assurance that the oxygen dissociation curve of cats' blood in the presence of methemoglobin showed the shift described for blood of other species of animals, the curves were determined for normal blood and for blood in which 75 to 85 per cent (taken here as 80 per cent) of the hemoglobin had been converted to methemoglobin. The general procedure used was that described by Peters and Van Slyke (9) employing a double tonometer system for equilibration; all determinations were made at 38° C and 35 to 38 mm carbon dioxide. The methemoglobin was formed by administering sodium nitrite to a cat and blood was obtained by heart puncture when the desired percentage of methemoglobin was reached. The curves obtained are given in figure 1. That for normal blood corresponds closely with the curve given by Starling (10). The curves indicated for hemoglobin in the presence of carbon monoxide hemoglobin were not determined directly but were calculated on the principle defined by Haldane (11, 12) as modified and simplified by Darling and Roughton. It will be observed that the shift in the dissociation curve caused by the presence of 80 per cent methemoglobin corresponds closely to that caused by 40 to 50 per cent carbon monoxide hemoglobin. One value determined for 49 per cent methemoglobin was close to that for 25 per cent carbon monoxide hemoglobin. The shift in the dissociation curve of the cat caused by methemoglobin is approximately half as great as that caused by carbon monoxide hemoglobin. For human hemoglobin, Darling

and Roughton found that 23 and 32 per cent carbon monoxide hemoglobin caused shifts corresponding closely to those from 43 per cent and 58 per cent methemoglobin.

THE PERCENTAGE OF CARBON MONOXIDE HEMOGLOBIN AT UNCONSCIOUSNESS AND DEATH IN THE CAT. Forty-two cats were exposed individually in a gassing chamber, of 1 cubic meter capacity, to concentrations of carbon monoxide ranging from 0.11 to 1.0 per cent and in 2 instances to 90 per cent. For prolonged exposures, extending to 47 hours, provision was made for the removal of carbon dioxide with soda lime and the addition of oxygen to maintain a normal atmos-

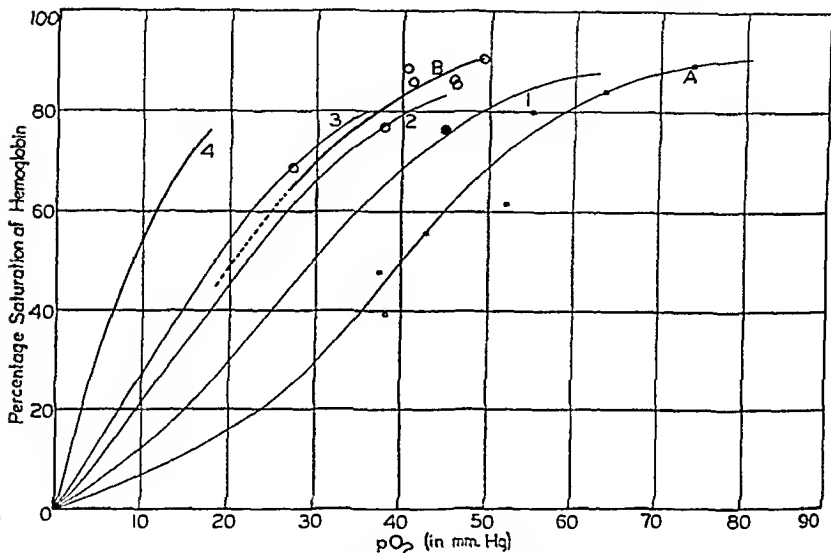


FIG. 1. OXYGEN DISSOCIATION CURVES OF THE CAT

Curve A . . . normal curve for cat. Rectangles (□) experimental points.

Curve B . . . curve in presence of 80 per cent methemoglobin.

Circles (○) = experimental points.

Black dot (●) = experimental point in presence of 49 per cent methemoglobin.

Curves 1, 2, 3 and 4 in presence of 20, 40, 50 and 80 per cent CO hemoglobin.

phere. A hand hole closed with an inserted rubber glove was provided for handling the animals during gassing. Twelve of the cats were removed at the moment when unconsciousness to the extent of abolition of corneal reflex had developed and 25 were allowed to die before removal. Blood was immediately drawn and the carbon monoxide content and capacity were determined either by the method of Van Slyke and Neill (13) and Van Slyke and Hiller (14) or that of Horvath and Roughton (15). The total hemoglobin for the various animals ranged from 9.9 to 24.1 volumes per cent. Figure 2 gives the findings for all of the animals which were rendered unconscious or died. In addition, 5 animals were kept at saturations of 50 to 59 per cent for 18 to 47 hours without becoming unconscious.

The values for animals which died during the first hour or hour and a half are not truly indicative of the saturation causing death. The rate of absorption of carbon monoxide from air containing 0.5 or more per cent of the gas is so rapid that appreciable amounts over and above that giving a saturation causing death are absorbed after respiratory failure has started. This is shown clearly in the animals breathing 90 per cent carbon monoxide in which death occurred at 100 per cent saturation in 3 to 6 minutes. Likewise, the single value obtained at unconsciousness for an animal breathing 0.5 per cent carbon monoxide cannot be taken as a valid indication of the saturation causing unconsciousness. This animal was exposed for only 20 minutes and a delay of 2 or 3 minutes in judging

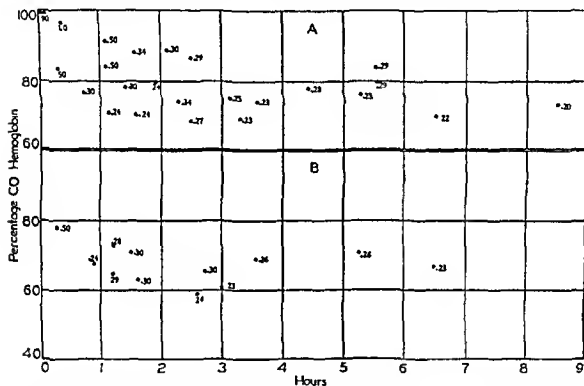


FIG 2 A CARBON MONOXIDE SATURATIONS AT DEATH IN CATS B CARBON MONOXIDE SATURATIONS AT UNCONSCIOUSNESS IN CATS

Dots represent time and percentage saturation and numbers beside dots indicate per cent CO in air

the time at which unconsciousness developed would lead to the accumulation of considerable carbon monoxide

Excluding the results obtained at these higher concentrations of carbon monoxide it would appear that the time of asphyxiation, up to $7\frac{1}{2}$ to $8\frac{1}{2}$ hours as employed here, had little influence on the saturation causing unconsciousness and death. The average saturation for death was 71 per cent with extremes of 68 and 88, and for unconsciousness 66 per cent with extremes of 59 and 73. In addition, as stated 5 animals did not develop unconsciousness after exposures of 18 and 47 hours with final saturations of 50 to 59 per cent.

The saturation at death found here corresponds to that given in the literature for most experimental animals. This is also the general range reported for human

beings in fatal accidental or suicidal asphyxiation. Gettler and Freimuth (16), however, from blood obtained at autopsy of victims dying in the site of the gassing, found that in 48.5 per cent of the instances the saturation was over 70 per cent; in 28 per cent, it was between 60 and 70; in 14.5 per cent, it was between 50 and 60; and in 9 per cent, it was below 50, being in one instance as low as 30 per cent. There are several possible explanations for the low values found in a quarter of these cases other than that which Gettler and Freimuth give, i.e., that 30 per cent saturation may be fatal to human beings. Some of the victims may have had severe cardiac disease and therefore had died of heart failure from moderate asphyxiation. No statement is made on this feature although autopsies were apparently performed. The second, and more probable, explanation

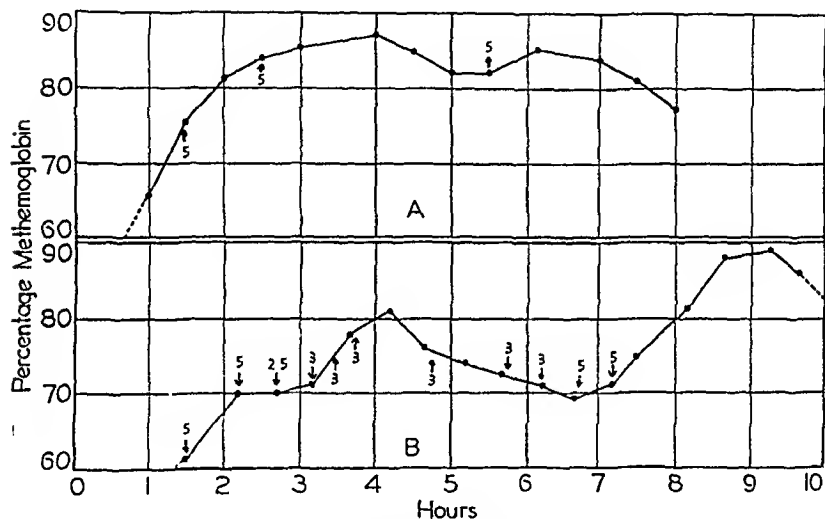


FIG. 3. A. CAT GIVEN INJECTIONS OF SODIUM NITRITE. B. DOG GIVEN INJECTIONS OF SODIUM NITRITE

Arrows indicate time of injection and numbers at arrows, dose in mg. per kg.

is that death may have been due to a high percentage saturation, but, in the instances recorded, the damage caused was survived for some time; that in this time the concentration of carbon monoxide in the air had fallen either because of diminished supply of gas or increased room ventilation with change of wind or outside temperature; and that in this interval the saturation of the blood had decreased but without saving life. It is not uncommon to have victims of severe gassing brought to the hospital while unconscious and subsequently die, but with sufficient lapse of time to allow a great part or even all of the carbon monoxide to leave the blood. The explanation given here seems particularly probable in cases 10 and 11 as reported by Gettler and Freimuth; in these, a man and woman were found together in a room, both dead. The blood of the woman who, if she were smaller than the man would probably succumb earlier, had a

saturation of 81.1 per cent and that of the man 33.6 per cent. The study of Gettler and Freimuth shows the saturations of the blood which were present at death, but it does not necessarily show the saturation responsible for death. There is no record of the concentration of carbon monoxide in the air at the time the fatalities were discovered.

ASPHYXIA FROM METHEMOGLOBINEMIA In a series of animals, both dogs and cats, sodium nitrite was injected intraperitoneally but in repeated small amounts to avoid accumulation of any large residuum of nitrite which might lower arterial pressure and correspondingly increase the arterial-venous oxygen difference to the circulation to the brain and so exacerbate asphyxia in this organ. The percentage conversion of methemoglobin was determined at frequent intervals. Figures 3a and 3b show the results from two experiments typical of many similar ones. In the first of these, a cat of 3.4 kg. was given initially 35 mg./kg. of sodium nitrite followed by doses of 5 mg./kg. at times indicated in figure 3a. At the end of 1 hour, the methemoglobin had risen to 66 per cent, the animal showed no ill effects and was able to rise and move in a manner apparently normal. At the end of 2 hours the methemoglobin had reached 82 per cent and the animal, although somewhat depressed, was conscious, on being forced to move it staggered slightly but did not become unconscious. At the end of 4 hours, the methemoglobin was 87.5 per cent and the animal was still conscious. The methemoglobin was maintained above 80 per cent for 3½ hours longer but neither unconsciousness nor any other striking symptom developed.

In the experiment from which the data for figure 3b were obtained, a dog of 4.6 kg. was given nitrite in an initial dose of 25 mg./kg. followed by doses of 2.5, 3.0 and 5.0 mg./kg. at times indicated in figure 3b. When the methemoglobin rose to 70 to 80 per cent, the animal was somewhat depressed but able to walk without staggering. Between 80 and 85 per cent, there was some ataxia but no unconsciousness. Between 85 and 89 per cent, the animal was prostrate but still conscious, on attempting to rise, there were periods of unconsciousness lasting for 30 seconds to 1 minute.

Judged from observation of the behavior of the animals, the asphyxia caused by 85 to 89 per cent methemoglobin corresponds to that induced by about 60 per cent carbon monoxide.

CONCLUSIONS

- (1) The shift in the dissociation curve of the blood caused by methemoglobin has been confirmed for cats. The shift is approximately one half that caused by carbon monoxide hemoglobin.
- (2) Cats asphyxiated with carbon monoxide became unconscious and died at average values of 66 and 71 per cent conversion of hemoglobin to carbon monoxide hemoglobin.
- (3) Cats and dogs can tolerate more than 80 per cent conversion of hemoglobin to methemoglobin without becoming unconscious.
- (4) The asphyxial effects of methemoglobin are significantly less than those of carbon monoxide hemoglobin.

REFERENCES

- (1) DARLINO AND ROUGHTON, Amer. J. Physiol., 137: 56, 1942.
- (2) HALDANE, MAKOILL AND MAVROGORDATO, J. Physiol., 21: 160, 1897.
- (3) VANDENBELT, Pfeiffer, Kaiser and Sibert, J. Pharmacol., 80: 31, 1944.
- (4) SAYERS AND YANT, Bureau Mines Report of Investigations 2476, 1923.
- (5) HENDERSON AND HAOGARD, J. Pharmacol. 16: 11, 1920.
- (6) LÓLLI, LESTER AND RUBIN, J. Pharmacol. 80: 74, 1944.
- (7) LESTER, GREENBERG AND SHUKOVSKY, J. Pharmacol. 80: 78, 1944.
- (8) GREENBERG, LESTER AND HAGOARD, J. Biol. Chem., 151: 665, 1943.
- (9) PETERS AND VAN SLYKE, Quantitative clinical chemistry, Vol. II, pp. 302, 319. Williams & Wilkins Co., Baltimore, 1932.
- (10) STARLING, Principles of human physiology. 3d Ed. p. 1136. J. & A. Churchill, London, 1920.
- (11) DOUGLAS, HALDANE AND HALDANE, J. Physiol. 44: 275, 1912.
- (12) HALDANE, J. Physiol. 45: xxii, 1912-13.
- (13) VAN SLYKE AND NEILL, J. Biol. Chem. 61: 523, 1924.
- (14) VAN SLYKE AND HILLER, J. Biol. Chem. 78: 807, 1928.
- (15) HORVATH AND ROUGHTON, J. Biol. Chem. 144: 747, 1942.
- (16) GETTLER AND FREIMUTH, Amer. J. Clin. Path. 10: 603, 1940.

CHEMOTHERAPY OF FILARIASIS IN THE COTTON RAT BY ADMINISTRATION OF NEOSTAM AND OF NEOSTIBOSAN

JAMES T. CULBERTSON AND HARRY M. ROSE

*From the Departments of Bacteriology and Medicine, College of Physicians and Surgeons,
Columbia University, New York*

Received for publication March 13, 1944

Cotton rats are frequently infected with a species of filarial worm known as *Latomosoides carini* (1). The adult worms live in the pleural space and microfilariae occur constantly in the peripheral blood of the rats. Because the infection bears, in some respects, a close similarity to certain of the human filariases, it seemed reasonable to the authors that the cotton rat infection might serve well as a testing medium in a search for drugs with potential action in the human filarial diseases. A number of drugs have already been tested in cotton rats in this laboratory. Two of these, neostam (stibamine glucoside, Burroughs Wellcome and Co.) and neostibosan (metachlor paracetaminophenyl stibiate of sodium) have been found particularly effective (2). The present paper describes the results of the administration of neostam and of neostibosan to a number of infected cotton rats. As will be seen following the administration of these drugs, the adult worms were killed in the pleural space and the microfilariae disappeared from the peripheral blood of infected animals. As will also be indicated, neostam and neostibosan manifested powerful action upon the adult worms when tested *in vitro*.

MATERIALS AND GENERAL PROCEDURES *The Animals Used* The infected cotton rats were obtained from Zoological Research Supply, Englewood, Florida. Their infections were natural, having been contracted in the field. Most of the cotton rats used weighed between 100 and 150 grams.

The Drugs Used The neostam was generously supplied by the Wellcome Research Laboratories, Tuckahoe, New York. The authors wish to thank Dr. E. E. Nelson, Director of Research, and Dr. E. J. DeBeer, Assistant Director of Research, for their interest in this work.

The neostibosan was provided by the Winthrop Chemical Company of New York City. The authors are grateful to Dr. J. B. Rice, Director, and Dr. C. B. McDermott, Associate Director of the Research Division of this company, for their cooperation.

Method of Treatment Those infected animals which were injected with neostam received 40 mg. of the drug (a few were given up to 60 mg.) approximately four times weekly, unless otherwise indicated. Because of the difficulty in restraining the rather vicious wild animals, it was found necessary to administer the drug intramuscularly in the thigh, although it was realized that greater effectiveness and less local irritation would probably have followed the intravenous route of treatment.

The infected animals which were treated with neostibosan received a series of 40 mg. doses and, after a brief rest period, a series of 80 mg. doses, as indicated in the footnote of table 4.

Estimation of Number of Microfilariae The number of microfilariae was estimated by counting those seen in 100 microscope fields ($\times 430$) of fresh tail blood under a cover slip. Counts were made practically every day after treatment was started.

Technic of Tests Performed in vitro. Adult filarial worms were removed aseptically from the pleural space of an untreated cotton rat and from six to ten individuals, including both males and females, were transferred to 50 cc. Erlenmeyer flasks which contained 10 cc. of modified Simms' balanced salt solution with 10 per cent horse serum, plus 0.1 per cent glucose with or without drug. The flasks were incubated at 37°C. At twenty-four-hour intervals the worms were examined carefully for survival, as shown by their continued movement.

TABLE 1

Effect of neostam on the filarial worm Litomosoides carinii in cotton rats

COTTON RAT NUMBER	MICROPILARIAE COUNTED IN 100 MICROSCOPE FIELDS (X430) ON DESIGNATED DAYS											NUMBER OF ADULTS RECOVERED AT AUTOPSY*
	Day before treat- ment	Days after treatment										
		1	7	14	21	28	35	42	49	56	64	
1	288	236	220	184	126	82	90	50	50	13	3†	40 to 50; dead; many matted together
2	136	94	100	52	20	28	16	7	5	2	0†	50 to 60; dead; some matted together
3	44	0	4	3	5	5	1	3	1	0†		several; dead; enveloped by exudate
4	50	28	32	22	24	28	4	1	3	1	0†	10; dead; matted together
5	4	4	0	0	0	0	0†					1; dead
6	276	116	96	70	144	120	50	44	20	8	1†	50; dead; matted together
7	12	10	0	0	0	0	0†					10; dead; enveloped by exu- date
8	92	62	92	70	38	7	6	6	0	3	0†	50; dead; some matted to- gether
9	32	32	38	3	14	2	2	0†				none
10	24	12	6	2	1	1	0†					none
11	180	152	230	84	16	64	8	3	2	1	0†	50; dead; matted together
12	6	8	18	2	0†							several; dead; enveloped by exudate
13	108	96	116	62	26	5	0†					40; dead; some matted to- gether
14	124	44	56	0	0	0†						20; dead; enveloped by exu- date
15	92	72	16	4	0	0	0†					25; dead; enveloped by exu- date
Control 16	16	18	36	12	24	20	10	38	48	42	52	8; living, moving
Control 17	252	232	232	176	110	192	90	176	136	186	198	50; living, moving

* Where worms are massed together, numbers are approximated.

† = Day of autopsy.

Schedule of treatment: Rats Nos. 1 through 12: 40 mgm. } 4 times weekly.
 Rats Nos. 13 through 15: 60 mgm. }
 Rats 16 and 17: untreated

EXPERIMENTAL RESULTS WITH NEOSTAM. *Effect of Drug in Filaria-infected Rats.* In the first experiment, fifteen infected cotton rats were treated with neostam for periods ranging from two to nine weeks. Several additional cotton rats were also examined on the same days as the treated animals but were left untreated as controls of the infection. In table 1 are given the schedule of

injections of the drug, the microfilaria counts on designated days, and the autopsy findings. It should be mentioned that the drug was withheld as soon as microfilariae disappeared from the blood.

In the case of every treated rat, the microfilaria count was sharply reduced. In thirteen of the fifteen treated animals, no microfilariae whatsoever could be observed after treatment and in the two remaining animals, the counts were extremely low. At autopsy, dead adult worms only were recovered from thirteen of the treated animals and none at all was found in the remaining two treated rats. The control, untreated rats, showed about the same number of microfilariae after nine weeks of observation as were presented initially and, at



FIG. 1. ADULT *LITOMOSOIDES CARINII* FROM THE PLEURAL SPACE OF INFECTED COTTON RATS TREATED WITH NEOSTAM
($\times 25$, photographed with top lighting)

autopsy, living, moving, adult worms were recovered from the pleural space of each

Effect of Drug on Adult Worms Recovered at Autopsy of Rats The adult worms which were recovered at autopsy of the treated animals were not only dead but also were usually held together in smaller or larger masses by a purulent necrotic exudate. This exudate was laid down initially along the length of individual worms, but, later, many worms with such exudate became matted together. Exudate continued to collect around these masses of worms, until the mass presented the appearance of being enveloped by it. In figure 1, photographs of several of these masses of worms from treated animals are shown, and, in figure 2, photomicrographs of sections of a typical mass from a treated rat as well as sections of normal worms from an untreated rat are presented.*

* The authors are pleased to acknowledge the kindness of Professor A. M. Pappenheimer of the Department of Pathology of this institution in preparing the sections and in interpreting them.

Effect of a Single Dose, or of a Small Number of Doses, of Drug.—Comparatively prolonged treatment was used in most of the animals presented in table 1. In order to determine whether such extended application of drug was necessary, a single dose (40 mgm.) of neostam was given to each of four infected animals and a total of three doses (each of 40 mgm.) was given during one week to six other infected rats. Following this treatment, all the animals were rested until autopsy, the tail blood being examined meanwhile for the presence of microfilariae.

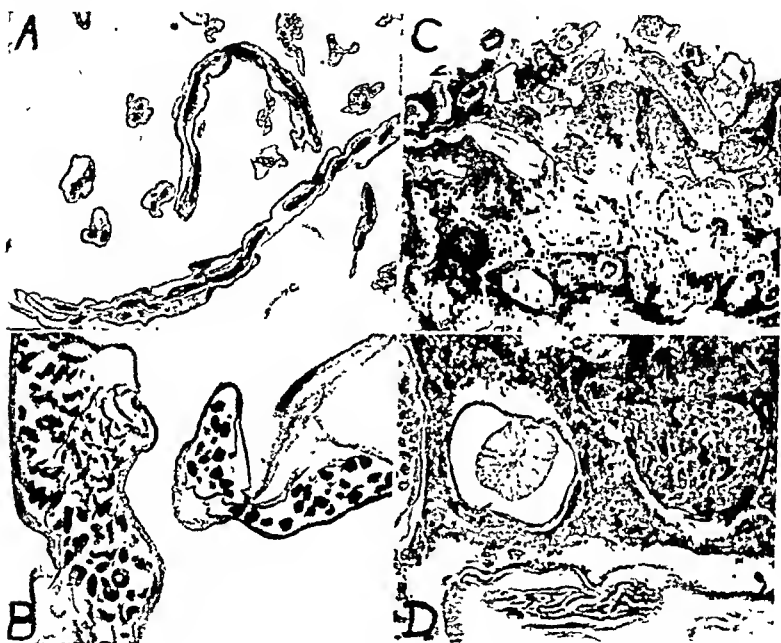


FIG. 2. A AND B: SECTIONS OF ADULT *LITOMOSOIDES CARINII* REMOVED FROM PLEURAL SPACE OF UNTREATED COTTON RATS

C AND D: SECTIONS OF ADULT *LITOMOSOIDES CARINII* REMOVED FROM PLEURAL SPACE OF COTTON RATS TREATED WITH NEOSTAM. (A AND C: $\times 55$; B AND D: $\times 300$.)

The four rats which received a single injection of drug were autopsied from 44 to 76 days after being treated. Every adult worm recovered at the autopsies was dead, and some of them were so degenerated as almost to be unrecognizable as filarial worms. All of them were covered with exudate or fat and gave evidence of having been dead for a considerable period. The microfilaria counts in all the rats had gradually declined until, by the day of autopsy, they had reached zero or a point where only an occasional microfilaria could be seen by prolonged search.

The six remaining rats, which had received three doses of drug within one week, were autopsied on the eleventh day after treatment had begun. At this

time, little or no change had occurred in the microfilaria counts. The adult worms, however, were all dead, massed together, and enveloped in exudate in four of the six rats. In the remaining two treated animals, some adult filarias were still alive and moving, although most of the adults in these animals, too, were dead. Evidently, as shown by the data in table 2, microfilariae will persist in the circulation of infected animals long after the adult parasites have been killed by neostam. It is indicated, thus, that the adult parasites are more easily affected by the drug than are the microfilariae.

TABLE 2

Effect on cotton rat filariasis of a short course of treatment with neostam

COTTON RAT NUMBER	DOSE OF DRUG	MICROFILARIAE COUNTED IN 100 FIELDS (X430) ON DESIGNATED DAYS												ADULT WORMS RECOVERED AT AUTOPSY*
		Day before treat- ment	Days After Initial dose of Drug											
			1	7	11	14	21	44	49	56	64	76		
1	Single dose of 40 mgm	18	10	7	4	12	2	0†					5, dead, some adherent exu- date	
		16	4	3	2	1	1	1	1	0†			2 or 3, dead, and degenerated, embedded in fat	
3		70	126	40	104	38	14	4	4	4	2	3†	15, dead and generated, in exu- date	
4		4	10	9	5	3	2	4	2	2	0†		3, dead, enveloped in fat	
5	Three doses, 40 mgm each, during one week	52	56	22	24†								20, dead, massed together in exudate	
6		6	20	26	16†								6, dead, massed together in exudate	
7		22	26	12	16†								12, 6 dead and in exudate, others living	
8		68	60	24	36†								30, 20 dead and in exudate, others living	
9		22	14	18	46†								12, dead, massed together in exudate	
10		38	54	38	42†								21, dead, massed together in exudate	

* Where worms are massed together numbers are estimated

† = day of autopsy

Effect of Drug in vitro In table 3 are presented the results of the *in vitro* tests. It is noted that whereas worms in the control flasks continued to move for at least four days (and usually well beyond one week), the worms exposed to even comparatively low concentrations of the drug—from 1 to 5 mg per cent—were dead by the fourth day. When higher concentrations of drug were used—e.g., 50 mg per cent—the adult filarial worms were dead within 24 hours. Evidently, neostam manifests a powerful direct filaricidal action upon the adult worms *in vitro*.

EXPERIMENTAL WORK WITH NEOSTIBOSAN *Effect of Drug in Filaria infected*

TABLE 3
Effect of neostam on adult Litomosoides carinii in vitro

NEOSTAM mg. %	OBSERVATIONS AFTER			
	24 hrs.	48 hrs.	72 hrs.	96 hrs.
50	dead			
25	sluggish	♂ active ♀ dead	dead	
10	active	♂ active ♀ sluggish	♂ active ♀ dead	dead
5	active	active	active	dead
1	active	active	active	♂ very sluggish ♀ dead
Control	active	active	active	active

Parasites transferred to 50 cc. Erlenmeyer flasks each containing 10 cc. of balanced salt solution plus 0.1 per cent glucose, with or without drug. Incubation at 37°C.

TABLE 4
Effect of neostibosan on filariasis of the cotton rat

COTTON RAT NUMBER	NUMBER OF MICROFILARIA SEEN IN 100 FIELDS OF MICROSCOPE (X 430)											RECOVERY OF ADULT FILARIAS AT AUTOPSY†
	Day treat- ment began	Days after treatment										
		2	7	14	21	41	48	55	62	72	81	
1	36	40	26	10	22	18	6	4	2	3	0*	4; all dead; enveloped by fat
2	186	64	50	44	42	28	6	9	1	2	0*	25; all dead; matted together in exudate
3	76	78	60	34	64	30	14	2	4	3	0*	30; all dead; matted together in exudate
4	140	266	200	66	74	84	54	26	20	42	24*	30; all dead; matted together in exudate
5	62	80	40	50*								25; 6 living; many covered with bloody exudate
6	4	0	0	0*								4; all dead; matted together in exudate
7	13	16	6	9*								20; all dead; matted together in exudate
8	30	10	13	4*								15; all dead; matted together in exudate
9	7	20	12	6*								10; all dead; matted together in exudate

* Day of autopsy.

† When worms are matted together, numbers are approximated.

Schedule of treatment, rats No. 1-4:

40 mg. of Neostibosan given intramuscularly on day treatment began and 2, 3, 5, 6, 7, 7, 8, 10, 12, and 15 days thereafter.

80 mg. of Neostibosan given 41, 43, 46, 48, 50, 54, 57, and 62 days after the initial dose of drug.

No treatment between the 15th and 41st days nor after the 62nd day.

Schedule of treatment, rats No. 5-9.

40 mg. of Neostibosan intramuscularly on alternate days until autopsy on the 14th day.

Rats. In table 4 are shown the results of treating 9 rats with neostibosan. Four of these animals were given comparatively prolonged treatment and, from 3, all microfilariae had disappeared when the animals were autopsied 81 days after the

initial dose of drug. Every adult parasite found at the autopsy of the four rats was dead. The appearance of the adult worms, which were matted together and covered with exudate (see fig. 3), was indistinguishable from that of the worms recovered from rats treated with neostam.

The result of treating five infected cotton rats with neostibosan on alternate days for two weeks is also shown in table 4. During this period of treatment the microfilaria counts were not significantly reduced. When the rats were autopsied, however, all the adult worms were dead and matted together in 4 of the animals. In the remaining 1 rat, six adult worms were still moving sluggishly.

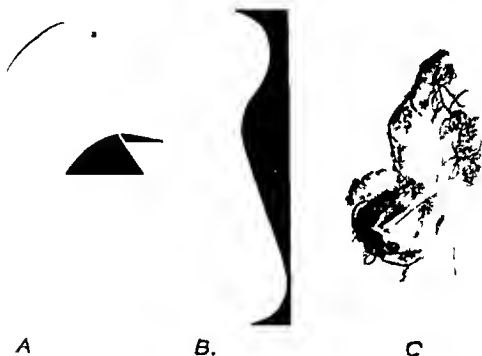


FIG. 3. ADULT *LITOMOSOIDES CARINII* FROM THE PLEURAL SPACE OF COTTON RATS. A (male worm) and B (female worm) from untreated rat. C, from rat treated with Neostibosan.

Evidently neostibosan has greater effectiveness in killing the adult worm than the microfilariae although, once the adult worms are killed, the number of microfilariae gradually declines.

Effect of Drug In Vitro. In vitro tests of neostibosan by the method already described gave results essentially similar to those obtained with neostam.

DISCUSSION. There seems no doubt from the data given that, as a result of the repeated injection of either neostam or neostibosan, adult filarial worms are killed in the pleural space and microfilariae finally disappear from the blood of infected cotton rats. These drugs appear to exert their principal effect on the adult worms, for microfilaria counts usually begin to decline only after treatment has continued for several days. In 1 number of the treated rats, microfilariae still were present in the peripheral blood on the day the rats were autopsied despite the fact that the adult worms which were then recovered from these animals presented evidence of having been dead for a considerable time.

The necrotic purulent exudate which was laid down around the worms probably represented a response by the animal to dead worms (acting as foreign bodies) after these had been killed by the drug. Many cells—leucocytes and round cells—could be seen in the exudate. Some treated animals from which only dead worms were recovered presented no exudate, the worms evidently having been killed for too brief a time prior to autopsy to permit the development of this response.

As yet, no tests to determine whether or not these drugs exert prophylactic action against filarial infection in cotton rats have been made. Such studies, as well as both therapeutic and prophylactic experiments in the filariases of man appear to offer good fields for future investigation.

CONCLUSION

The repeated injection of neostam or of neostibosan to cotton rats infected with the filarial worm *Litomosoides carinii* kills the adult parasites which occur in the pleural space of the animals and leads to the gradual disappearance of microfilariae from the peripheral blood. Even a single dose (40 mgm.) of one of these drugs (neostam) has proved sufficient to eliminate the parasite from some animals.

The adult parasites appear to be decidedly more susceptible to the action of neostam or of neostibosan than are the microfilariae, for within two weeks from the beginning of treatment, the adult worms are usually dead although the numbers of microfilaria in the tail blood at this time are essentially the same as before treatment began.

Neostam and neostibosan in concentrations of from 1 to 5 mg. per cent or more kill adult *Litomosoides carinii* *in vitro* after approximately four days at 37°C.

REFERENCES

- (1) Vaz, Z., *Ackertia* gen. nov. for *Litomosa burgoise* de la Barrera, 1926, with notes on the Synonymy and Morphological Variants of *Litomosoides carinii*, Travassos, 1919.
- (2) For an excellent summary to 1938 of the therapeutic activity of antimony, see SCHMIDT, H., AND PETER, F. M., *Advances in the Therapeutics of Antimony*, Leipzig, Thieme, 1938.

RELATIONSHIP OF CHEMICAL STRUCTURE OF SYMPATHOMIMETIC AMINES TO VENTRICULAR TACHYCARDIA DURING CYCLOPROPANE ANESTHESIA¹

O S ORTH, J W STUTZMAN AND WALTER J MEEK

From the Departments of Pharmacology and Physiology, University of Wisconsin Medical School, Madison

Received for publication March 27, 1944

In previous studies with comparable pressor dosages of eleven sympathomimetic amines, the five primary and secondary amines with a catechol nucleus caused ventricular tachycardia during cyclopropane anesthesia, and the one tertiary amine with a catechol nucleus did not (1, 2). Further studies have been made as 15 additional amines have become available. Tests of the present drugs have confirmed the conclusion that a catechol nucleus and also a primary or secondary amino group are necessary for the production of ventricular tachycardia by drugs in the dog during cyclopropane anesthesia. Further information regarding the importance of the structure of the amino group and the effect of the change of blood pressure on such tachycardia has been obtained.

METHODS The methods used were those employed in previous investigations (1). Dogs served as the test animal and were maintained in a known, constant plane of cyclopropane anesthesia throughout each experiment. The volume of fluid containing the amine and the time in which it was injected intravenously were kept constant, 5 cc being given at a steady rate in 50 seconds. For each drug direct determinations of blood pressure were made on several animals. In this way the equivalence of blood pressure rise as compared to that caused by the standard dose of 0.01 mgm of adrenalin per kilogram of body weight was determined and comparison made to similar data in the literature. Only one drug was compared with adrenalin on any animal in any day. All electrocardiographic observations and tracings were made using lead II with the animal lying on its left side.

RESULTS The amines used, the amount of each required to produce a rise in blood pressure equivalent to that of the standard adrenalin dosage, and their ability to cause ventricular tachycardia during cyclopropane anesthesia are indicated in table 1. They are also grouped with regard to their chemical similarity. The various cardiac arrhythmias resulting from injection of the amines are shown in table 2. The control results were those obtained when the amine was injected into the unanesthetized animal. The same dose of the amine was given on a different day when the animal had been equilibrated with a 30 to 32 per cent cyclopropane mixture by breathing it for 20 to 30 minutes. This concentration is sufficient to maintain dogs in surgical anesthesia with at least partial intercostal paralysis.

In the present series, 34 animals were tested and 56 adrenalin injections were

¹ Aided by a grant from the Wisconsin Alumni Research Foundation and The W. S. Merrell Company.

made. There were two animals that failed to have ventricular tachycardia when so tested. For any animal the duration of tachycardia on different days is practically constant. Six of the animals were lost from ventricular fibrillation, one when only 2 cc. of the test adrenalin dosage had been given, the other five animals with the full 5 cc. injection. No adrenalin injections were done with

TABLE 1

Tabulation of the drugs used in this study with the chemical structure, the equivalent pressor dosage of each, and the number of times that ventricular tachycardia was elicited from the animals indicated as being injected with each drug

DRUG							DOSAGE*	ANIMALS WITH VENTRICULAR TACHYCARDIA
							mg./kg.	
1	OH	OH	OH	H	H	CH ₃	0.01	32 of 34
2	OH	OH	=O	H	H	CH ₃	1.0	2 of 3
3	OH	OH	H	H	H ₂		0.125	4 of 5
4	OH	OH	H	H	(CH ₃) ₂		0.4	0 of 4
5	OH	OH	=O	H	(CH ₃) ₂		30-40	1 of 5
6	OH	OH	H	CH ₃	H ₂		0.35	3 of 3
7	OH	OH	=O	CH ₃	H ₂		3.75	4 of 4
8	OH	OH	H	CH ₃	H	CH ₃	(0.35) 1.00	2 of 3 ♂ Bl. pr. rise 3 of 5 ♂ Bl. pr. rise
9	OH	H	=O	CH ₃	H ₂		7.0	2 of 4
10	OH	H	OH	CH ₃	H	CH ₃	0.9-1.0	0 of 4
11	H	H	OH	CH ₃	CH ₃ C ₂ H ₅		>75	0 of 5
12	OH	H	OH	CH ₃	CH ₃ C ₂ H ₅		>75	0 of 4
13	OCOOC ₂ H ₅ OCOOC ₂ H ₅		H	H	(CH ₃) ₂		0.3-0.6	0 of 4
14	H	H	H	CH ₃	H ₂		7.7	0 of 5
15	H	H	CH ₃	H	H ₂		6.25	2 of 5
16	H	H	CH ₃	H	H	CH ₃	6.25	0 of 4
17	H	H	OH CH ₃	CH ₃	H ₂		6.25	0 of 5

* Amount required to produce a rise in blood pressure equivalent to that of the standard adrenalin dosage.

conscious animals since the previous studies had shown the dosage here used does not cause ventricular tachycardia in the unanesthetized animal.

DISCUSSION. Among the 17 amines in this study there were 8 with a catechol nucleus. Primary amines were No. 3 [α -(3,4-dihydroxyphenyl) β -amino ethane], No. 6 [α -(3,4-dihydroxyphenyl) β -amino propane], and No. 7 [α -(3,4-dihydroxyphenyl) α -keto β -amino propane]. Secondary amines were No. 1, adrenalin [α -(3,4-dihydroxyphenyl) β -methylamino ethanol], No. 2 adrenalin ketone (or kephrine), and No. 8 [α -(3,4-dihydroxyphenyl) β -methyl-amino

TABLE 2

Cardiac arrhythmias during surgical cyclopropane anesthesia resulting from the injection of blood pressure raising drugs in doses equal in effectiveness to 0.01 mgm. of adrenalin per kilogram

DRUG NUM BER	NAME OF DRUG AND PROCEDURE	DOSAGE	NUMBER OF ANIMALS	SINO-AURICULAR BLOCK	SINO-AURICULAR COUPLING	SINO-AURICULAR TACHYCARDIA	AURICULO VENTRICULAR NODAL BLOCK	A V NODAL EXTRASYSTOLES	A V NODAL RHYTHM	VENTRICULAR EXTRASYSTOLES	SLOW VENTRICULAR RHYTHM	VENTRICULAR TACHYCARDIA
		mgm/kg										
1	Adrenalin α (3,4-dihydroxyphenyl) β methylamino ethanol With cyclopropane	0.01	34	0	0	0	2	13	26	27	0	32
2	Adrenalone α (3,4-dihydroxyphenyl) α -keto β methylamino ethane Unanesthetized With cyclopropane	1.0	3 3	0 0	0 0	0 0	2 1	3 2	2 3	0 1	0 0	0 2
3	α (3,4-dihydroxyphenyl) β amino ethane Unanesthetized With cyclopropane	0.125	3 5	0 0	0 0	0 0	1 0	2 2	2 4	2 5	1 1	0 4
4	α (3,4-dihydroxyphenyl) β dimethylamino ethane With cyclopropane	0.4	4	0	0	0	1	0	1	2	1	0
5	α (3,4-dihydroxyphenyl) α -keto β -dimethylamino ethane Unanesthetized With cyclopropane	30-40	5 5	0 0	0 0	0 0	2 1	2 1	0 1	0 1	0 0	0 1
6	α (3,4-dihydroxyphenyl) β amino propane Unanesthetized With cyclopropane	0.35	3 3	0 0	0 0	0 0	2 0	2 0	2 1	0 2	2 1	0 3
7	α (3,4-dihydroxyphenyl) α -keto β -amino propane Unanesthetized With cyclopropane	3.75	4 4	0 0	0 0	0 0	3 0	3 1	2 0	2 3	1 0	2 4
8	α (3,4-dihydroxyphenyl) β -methylamino propane Unanesthetized With cyclopropane	0.35 *(0.35) 1.0	3 3 5	1 0 0	0 0 0	0 0 0	0 0 0	2 0 0	0 2 5	1 1 2	1 0 2	0 2 3
9	α (4-hydroxyphenyl) α -keto β -amino propane Unanesthetized With cyclopropane	7.0	3 4	1 0	0 0	0 3	1 0	3 0	1 1	0 0	0 0	0 2
10	α (4-hydroxyphenyl) β methylamino propanol Unanesthetized With cyclopropane	0.9	4 4	0 0	0 0	0 3	0 0	1 0	0 0	1 2	1 0	0 0

TABLE 2—Continued

DRUG NUM- BER	NAME OF DRUG AND PROCEDURE	DOSAGE	NUMBER OF ANIMALS	SINO-AURICULAR BLOCK	SINO-AURICULAR COUPLING	SINO-AURICULAR TACHYCARDIA	AURICULO-VENTRICULAR NODAL BLOCK	A-V NODAL EXTRASYSTOLES	A-V NODAL RHYTHM	VENTRICULAR EXTRASYSTOLES	SLOW VENTRICULAR RHYTHM	VENTRICULAR TACHYCARDIA
		mgm./kg.										
11	α phenyl β , methyl, ethyl amino propanol Unanesthetized With cyclopropane	51 51-75	2 5	0 0	0 0	0 1	0 0	0 0	0 3	0 1	0 1	0 0
12	α (4-hydroxyphenyl) β , methyl, ethyl amino propanol Unanesthetized With cyclopropane	35 25-75	1 4	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0
13	α (3,4-di[ethylcarbonyldioxy]phenyl) β dimethyl amino ethane With cyclopropane	0.3-1.6	4	0	0	0	0	0	0	0	0	0
14	α phenyl β amino isobutane Unanesthetized With cyclopropane	7.7	5 5	0 0	0 0	0 5	1 0	1 0	0 3	1 2	0 0	0 0
15	α amino β phenyl propane Unanesthetized With cyclopropane	6.25	6 5	0 0	0 0	0 3	1 0	4 1	2 3	1 0	1 0	0 2
16	α methylamino β phenyl propane Unanesthetized With cyclopropane	6.25	4 4	1 0	1 0	0 4	0 0	1 0	1 0	0 0	0 0	0 0
17	α amino γ phenyl γ butanol Unanesthetized With cyclopropane	6.25	5 5	1 0	1 0	0 5	2 0	2 1	0 2	1 3	0 1	0 0

* No rise in blood pressure with 0.35 mgm./kg. but ventricular tachycardia occurred in two animals.

propane]. Tertiary amines tested were No. 4 [α -(3,4-dihydroxyphenyl) β -dimethylamino ethane] and No. 5 [α -(3,4-dihydroxyphenyl) α -keto β -dimethylamino ethane]. Since crystalline adrenalone was available, repetition of a previous study (1) of it was made. The generally available amines possessing a catechol nucleus have now been tested.

From the tabulated data it will be noted that all the primary and secondary amines possessing a catechol nucleus were effective in producing ventricular tachycardia when administered during cyclopropane anesthesia, as previously concluded (1). Such a result was particularly striking with No. 8, which in the expected equivalent pressor dose of 0.35 mgm. per kilogram caused a fall rather than a rise in blood pressure and yet produced ventricular tachycardia in 2 of 3 animals. With a dose of 1.0 mgm. there averaged a 36 mm. fall, then a 76 mm.

Hg rise above the previous control level. Alles found somewhat similar effects, although not exactly the same ratio of pressor activity, in his initial work after synthesizing this drug (3). The rise in pressure obtained was equivalent to that of the preceding and succeeding adrenalin injection changes. This is additional proof to that given previously by Allen, Stutzman and Meek (4) that neither height nor angle of rise in blood pressure is a dominant factor in the production of ventricular tachycardia, as claimed by Shen (5, 6).

Neither of the two catechol nucleus tertiary amines, Nos. 4 and 5, consistently produced ventricular tachycardia although it did occur in one experiment with the latter drug. The remaining nine amines tested did not possess a catechol nucleus and in comparable pressor dosages did not generally cause ventricular tachycardia.

That most of the amines in large enough dosages will elicit tachycardia was shown again when several drugs were given in excessive amounts during the determination of pressor dosages equivalent to the adrenalin standard. Among those in which comparable blood pressure raising dosage varied from previous reports were No. 4 [α (3,4-dihydroxyphenyl) β dimethylamino ethane], No. 10 [α (4 hydroxyphenyl) β methylamino propanol], No. 11 [α phenyl β methyl, ethyl amino propanol], No. 12 [α (4 hydroxyphenyl) β methyl, ethyl amino propanol], and No. 13 [α (3,4 di[ethylcarbonyldioxy]phenyl) β dimethylamino ethane]. The ratios determined on spinal dogs for Nos. 11 and 12 were reported as 75 and 25 mgm. per kilogram (7). In our intact animals these doses did not cause rises equivalent to the adrenalin standard. When one animal was tested with 75 mgm. of No. 11 per kilogram, progressive cardiac depression and death resulted in 10 minutes. Subsequent administrations of the amine were on a 51 mgm. basis. The control tests at the lower dosage elicited convulsive seizures of 30 and 40 seconds duration in two animals, but both recovered. Four animals were given 25, 35, 37.5, and 75 mgm. of No. 12 per kilogram, and it elicited rises of 40, 38, 36, and 20 mm. Hg, respectively, hence further increase of dosage was not indicated. No cardiac irregularities were produced by this amine on the control or during the tests with cyclopropane anesthesia. Both of these amines in such high dosages caused so much respiratory depression that the animal was disconnected from the anesthetic supply and artificial respiration was given.

Possible explanatory factors for the disparity in pressor dosages found in published data are such variations as (a) use of intact animals versus spinal preparations, (b) use of a different species, (c) use of different anesthetic agents, (d) lack of a controlled and known depth of anesthesia, (e) variation in route and rate of administration of drugs, (f) administration of unphysiological amounts of drugs, and particularly (g) testing with several drugs in the same animal on the same day without due regard to summation, potentiation or tachyphylaxis or control comparisons (as with adrenalin) between injections. To avoid such complications in the present study only one amine was compared against adrenalin in any day, and if tachyphylaxis was known or found to occur only one administration of the drug was made in a day. For repeated testing in the same

animal it has been possible to use more than one dose of a drug, or several drugs, by cannulation of a femoral or carotid artery after shaving and cleansing the skin with tincture of iodine, then dusting sulfanilamide or sulfathiazole powder into the cannulation wound. At the completion of the experiment the artery was ligated, more of the chemotherapeutic agent applied, and the animal allowed one to two or ten to fourteen days to recover. Intermediate recovery time may lead to rupture of the artery if there is any struggling during an attempted anesthetic induction. Blood pressure rises from the standard adrenalin injection check within 10 mm. Hg in successive experiments *provided* the same depth of anesthesia is maintained. Since the blood pressure rises were comparable to those obtained by massage of the adrenal glands or stimulation of the splanchnic nerves, they are considered to be within possibly occurring physiological limits.

CONCLUSION

Fifteen additional sympathomimetic amines have been tested for their ability to produce ventricular tachycardia in the dog during cyclopropane anesthesia. In a dosage producing a blood pressure rise equal to that caused by 0.01 mgm. of adrenalin per kilogram, the six primary and secondary amines with a catechol nucleus consistently elicited ventricular tachycardia. Tertiary amines with a catechol nucleus and the other amines used did not cause this irregularity.

We are indebted for the supply of drugs used in this study, as follows: to Dr. Gordon A. Alles of Pasadena, California for Nos. 3, 6, 8; to The Lakeside Laboratories, Inc. of Milwaukee for No. 5; to The Wm. S. Merrell Company for Nos. 9, 11, 12, 14, 15, 16, 17; to The Burroughs Welleome Company for Nos. 2, 4, 13, and to The Winthrop Chemical Company for Nos. 7 and 10.

REFERENCES

- (1) ORTH, O. S., M. D. LEIGH, C. H. MELLISH AND J. W. STUTZMAN, Action of sympathomimetic amines in cyclopropane, ether and chloroform anesthesia. *THIS JOURNAL*, 67: 1 (Sept.) 1939.
- (2) STUTZMAN, J. W., AND O. S. ORTH, Studies of the actions of methyl-adrenalin; methadren. *THIS JOURNAL*, 69: 1 (May) 1940.
- (3) Personal communication from Dr. Gordon A. Alles.
- (4) ALLEN, C. R., J. W. STUTZMAN AND W. J. MEEK, The production of ventricular tachycardia by adrenalin in cyclopropane anesthesia. *Anesthesiology*, 1: 158 (Sept.) 1940.
- (5) SHEN, T. C. R., The protective action of piperido-methyl-3-benzodioxine (F 933) diethyl-amino-methyl-3-benzodioxane (F 883) and yohimbine upon the chloroform adrenalin ventricular fibrillation. *Arch. Internat. Pharmacodyn. et de Therap.*, 59: 243 (June) 1938.
- (6) SHEN, T. C. R., AND R. MARRI, Further studies on ventricular fibrillation. *Arch. Internat. Pharmacodyn. et de Therap.*, 64: 53 (Feb.) 1940.
- (7) Personal communication from Dr. R. S. Shelton of The Wm. S. Merrell Company.

AN EVALUATION OF THE INFLUENCE OF SUCCINATE AND MALONATE ON BARBITURATE HYPNOSIS

KARL H. BEYER AND ALBERT R. LATVEN

From the Department of Pharmacology, The Medical-Research Division, Sharp and Dohme, Inc., Glenolden, Pa.

Received for publication March 29, 1944

The ability of the brain to oxidize glucose, lactate and pyruvate *in vitro* has been shown to be inhibited markedly by certain barbituric acid derivatives (1). The oxidation of succinate or of indophenol was not inhibited and glutamate oxidation was only partially inhibited under the above conditions. In that same publication Quastel and Wheatley presented data showing that there was good agreement between the effectiveness of a barbiturate as a hypnotic agent and its ability to inhibit the over-all oxygen consumption of brain tissue *in vitro*. Recently Fuhrman and Field (2) studied more thoroughly the relation of structure to the ability of barbituric acid derivatives to inhibit the oxygen consumption of rat brain cortex. They found a close correlation, within a given series of both alkyl-ethyl and alkyl-allyl derivatives, between the amount of a compound necessary to produce fifty per cent inhibition of oxygen uptake by brain, the delay in onset of anesthesia and the generally recognized duration of hypnotic action of the compounds.

It seems well established from the above reports that barbiturates do inhibit respiration of the brain cortex to an extent more or less consistent with their potency as hypnotics. Quastel and Wheatley (1) went further to interpret hypnosis produced by these compounds as being due to an interference at cell interfaces with a mechanism which results in activation of the molecules of lactic and pyruvic acid.

It remained for Soskin and Taubenhaus (3) to reason, since barbiturates did not inhibit the oxidation of succinate, that "by supplying sufficient of the latter substrate (succinate) one might adequately maintain the metabolism of the brain of a poisoned animal until the barbiturate had been destroyed or excreted." The results of their experimentation substantiated their hypothesis. This correlation of information concerning the effects of compounds on intermediary cellular metabolism with the observed pharmacodynamic action of barbiturates apparently served at once to demonstrate the practicality of this approach to such a problem and to stimulate interest in sodium succinate as an antidote for barbiturate poisoning.

The purpose of the work presented in this paper was to evaluate in another laboratory the effectiveness of succinate therapy in shortening the duration of barbiturate hypnosis. That we were not able to obtain striking confirmation of the report referred to above does not invalidate the basic research on which the hypothesis was founded.

EXPERIMENTAL. The mice used in these experiments were healthy animals of a single strain weighing between 20 and 25 grams. Each animal was weighed to the nearest 0.1 gram and the doses were calculated on a mgm./kgm. basis. Sodium pentobarbital was injected intraperitoneally in a dose of 80 mgm./kgm. which produced hypnosis in all the animals. A single stock solution was used in all the experiments.

Immediately following the production of hypnosis (loss of the righting reflex) each mouse was given the compound being investigated by the intramuscular injection of 0.1 cc. of solution into one of the hind legs. The mice were observed constantly to determine when they had essentially recovered their righting reflexes. Three different experiments were performed on three groups of mice. None of the mice was used for more than a single determination. The amounts of sodium succinate used for the tests varied from 150 mgm./kgm. to 1,000 mgm./kgm. The dose of sodium malonate and of sodium glutamate was 250 mgm./kgm. The design of the experiments is apparent in table 1.

The rats used in these experiments were healthy animals of a single strain weighing 200 to 300 grams. In order to obtain uniform results all the animals were fasted over night preceding dosage; free access to water was permitted. Sodium pentobarbital was administered intraperitoneally in a dose of 30 mgm./kgm. from a single stock solution. The data on the rats were summarized in table 2.

In experiment I the rats were divided into 3 groups. On the first day of the experiment group A received pentobarbital alone, group B received pentobarbital plus malonate and group C received pentobarbital plus succinate. One week later the experiment was repeated. This time group A received pentobarbital plus sodium succinate, group B received only pentobarbital and group C received pentobarbital plus sodium malonate. In table 2 the data are analysed as cross-over experiments within each group of rats.

Experiment II was designed as a triple cross-over of a group of 20 rats divided into 3 groups of 7, 7 and 6 rats. The order of intramuscular injection of the compounds to be studied was rotated at weekly intervals so that at the end of 3 weeks each of the 20 rats had received (in addition to pentobarbital) 0.2 cc. of distilled water, 500 mgm./kgm. of sodium succinate and 500 mgm./kgm. of sodium malonate. However, in any one test $\frac{1}{3}$ of the group received succinate, $\frac{1}{3}$ received malonate and $\frac{1}{3}$ received distilled water in addition to pentobarbital.

Experiment III consisted of 7 rats which received sodium pentobarbital at 3 weekly intervals concomitantly with experiment II. The data on duration of hypnosis in these animals were analysed as a triple cross-over experiment in order to demonstrate the degree of variation inherent in such a procedure.

Since all the statistical methods may not be self evident the formulas used were as follows: Mean duration of hypnosis in minutes = M ; Standard deviation = $\sigma = \sqrt{\frac{\sum d^2}{(N-1)}}$;

Standard error = $\epsilon = \frac{\sigma}{\sqrt{n}}$; Significant difference = $t = \frac{M_1 - M_2}{\sqrt{\epsilon_1^2 + \epsilon_2^2}}$; probability (P) is expressed as the number of times the observation might occur due to chance in 100 trials, from tables of probability making use of t and n values.

The effect of sodium pentobarbital on the oxidation of succinate by the isolated succinoxidase system was determined with the aid of a conventional Warburg apparatus. Each flask contained:

- 0.3 cc. Na-K phosphate buffer M/4, pH 7.48
- 0.3 cc. Cytochrome C 1.0×10^{-7} mols./cc.
- 0.1 cc. CaCl_2 M/1000
- 0.1 cc. AlCl_3 M/1000
- 0.4 cc. 10 per cent mouse liver homogenate in phosphate buffer M/40, pH 7.48
- 0.3 cc. Sodium succinate M/2 (in side arm)
- 0.3 cc. Sodium pentobarbital, 1 per cent solution (or water for control)
- 1.2 cc. Distilled water

Cytochrome C was prepared essentially according to the method of Keilin and Hartree (4), stored in the lyophilized form and restored to the desired concentration at the time of use (5). The very dilute rat liver homogenate was used as a source of succinic dehydrogenase and cytochrome oxidase. The advantages of such an homogenate in this system have been discussed by Potter and Elvehjem (6). The succinate was tipped from the side arm into the contents of the flask following a 10 minute period of shaking in the water bath to permit temperature equilibration. The flasks were oscillated at a rate of 100 per minute through a stroke of 4 cm. Temperature = 38.6°C. Ten per cent NaOH saturated filter paper was contained in the center wells.

RESULTS Table 1 summarizes all the data obtained on the duration of pentobarbital induced hypnosis in mice as influenced by succinate, malonate or glutamate. In general, a *t* value of 2 or over indicates a statistically significant

TABLE 1

The influence of sodium succinate, sodium malonate and sodium glutamate on the duration of hypnosis induced in mice by sodium pentobarbital

EXPERIMENT	NO. OF MICE	NA PENTOBARBITAL I.P. DOSE mgm / kgm	INJECTION SUBSEQUENT TO LOSS OF RIGHTING REFLEX		ANALYSIS OF DURATION OF NARCOSIS*				
			Compound	I.M. Dose	<i>M</i>	σ	<i>s</i>	<i>t</i>	<i>P</i>
				mgm / kgm	minutes				
A	19	80	Control		91	26	5.9		
	18	80	Na succinate	150	74	16	3.8	2.41	2
B	10	80	Control		73	20	4.8		
	20	80	Na succinate	150	69	11	2.5	0.77	45
	10	80	Na malonate	250	63	17	3.9	1.66	40
	10	80	Na glutamate	250	66	16	3.7	1.10	25
C	16	80	Control		77	13	3.3		
	19	80	Na succinate	1000	53	14	3.2	5.25	<1

* *M* = mean duration in minutes, σ = standard deviation, *s* = standard error, *t* = significant difference, *P* = probability, in 100 trials, of the difference from the control being due to chance. I.P. = intraperitoneal, I.M. = intramuscular injection.

difference between the control values and the duration of hypnosis as influenced by an agent. Also, it is unlikely that a result falling beyond a 5 per cent level of significance (*P* > 5) can be attributed to an unequivocal analeptic effect of the compound being studied.

Using these limits of significant differences it appears that an intramuscular dose of 150 mgm of sodium succinate per kgm did not consistently shorten the duration of pentobarbital hypnosis in the two groups of mice studied. Moreover, it should be noted that in experiment A, where the greatest effect of succinate was observed, the duration of hypnosis for the control group was much longer than for any other control or treated group of mice. When sodium succinate was injected in a dose of 1 gram/kgm the duration of hypnosis was significantly reduced (*t* = 5.25, *P* = < 1).

Neither malonate nor glutamate produced a marked effect on the duration

of pentobarbital hypnosis in mice. Also, from the data in table 2 it may be concluded that sodium malonate (250 to 500 mgm./kgm.) did not influence significantly the duration of pentobarbital hypnosis in rats.

Sodium succinate injected intramuscularly in doses of 250 and 500 mgm./kgm. decreased the duration of pentobarbital hypnosis in rats to about the same extent, judging from the *t* and *P* values presented in table 2. While this diminution was not great the injection of sodium succinate did decrease the duration of hypnosis under these conditions.

TABLE 2

The influence of sodium succinate and sodium malonate on the duration of hypnosis induced in rats by sodium pentobarbital

EXPERIMENT	NO. OF RATS	NA PENTOBARBITAL I.P. DOSE mgm./kgm.	INJECTION SUBSEQUENT TO LOSS OF RIGHTING REFLEX		ANALYSIS OF DURATION OF NARCOSIS*				
			Compound	I.M. dose mgm./kgm.	<i>M</i>	σ	ϵ	<i>t</i>	<i>P</i>
I _A	7	30	Control		52	5	1.9		
		30	Na succinate	250	38	15	5.6	2.34	4
I _B	8	30	Na malonate	250	88	39	13.7	0.33	75
		30	Control		81	45	15.9		
I _C	7	30	Na succinate	250	42	11	4.2	2.32	4
		30	Na malonate	250	75	36	13.6		
II	20	30	Distilled H ₂ O		83	19	4.3		
		30	Na succinate	500	69	18	4.0	2.40	2
		30	Na malonate	500	77	25	5.6	0.86	40
III	7	30	Control		81	27	10.2		
		30	Control		85	25	9.5	0.29	75
		30	Control		79	23	8.7	0.15	88

* *M* = mean duration in minutes; σ = standard deviation; ϵ = standard error; *t* = significant difference; *P* = probability of occurrence of the difference from the control being due to chance in 100 trials. I.P. = intraperitoneal; I.M. = intramuscular injection.

The data presented in experiment III, table 2, illustrate strikingly the minimal variation inherent in the duration of barbiturate hypnosis of the control animals when the procedure was repeated three times in a single group of rats and the data analysed as a triple cross-over experiment.

DISCUSSION. We have been able to confirm qualitatively the observation of Soskin and Taubenhau (3) that the intramuscular injection of sodium succinate decreased the duration of pentobarbital hypnosis in rats and also in mice. However, under the conditions of our experimentation the magnitude of this inhibitory effect was not nearly as great at any dosage level for succinate as they reported. Using data on the rat only, their *t* values at dosage levels of 250 and 500 mgm./kgm. were 5.5 and 7.9 respectively whereas ours were 2.34 and 2.4

respectively. The cause of this difference is not at once apparent since the details of the procedure in the two laboratories appear to be essentially similar.

The fundamental observations of Quastel and Wheatley (1) on which the succinate-barbiturate antagonism premise has been based are probably correct. We have repeated what appears to be the keystone of these observations pertaining to the present study, using what may be considered an isolated, complete succinoxidase system, and have found that the oxidation of succinate by this system was not inhibited by pentobarbital, table 3.

However, we believe that invoking these findings to explain a succinate-barbiturate antagonism limits too narrowly the possible modes of action of barbiturates on cellular respiration. Superficially, it would seem that if succinate could shorten pentobarbital hypnosis, malonate, by inhibiting the utilization of what succinate is present or produced in the body, might prolong hyp-

TABLE 3

Oxygen uptake in the course of the oxidation of succinate by the succinoxidase system in the absence and presence of pentobarbital

TIME	CONTROL	PENTOBARBITAL PRESENT*
MIN	CM MIN	CM MIN
0		
30	257	252
60	399	399

* Final concentration of sodium pentobarbital = 0.1 per cent

nosis. Also, the fact that malonic acid and urea combine to make up the barbiturate acid basic structure tempts one to examine the data even more closely for some reciprocal relationship in the action of succinate and malonate on barbiturate hypnosis. We have not found such a relationship to exist.

So much remains to be learned concerning the effect of barbiturates on cellular respiration and metabolism that it seems too early to consider the mode of action of these compounds as settled. It is to be hoped that the work of Soskin and Taubenhaus will stimulate further inquiry into the problem of what appears to be a quantitatively not too striking antagonism between pentobarbital and succinate.

SUMMARY

Sodium malonate and glutamate, as administered in these experiments, were without effect on the duration of pentobarbital hypnosis in mice or rats. We were able to confirm the observation that pentobarbital did not inhibit the *in vitro* oxidation of succinate by the succinoxidase system. Though it was found that the intramuscular administration of sodium succinate to mice and rats moderately diminished the duration of pentobarbital hypnosis, this succinate-barbiturate antagonism was not nearly as great on a dosage basis as has been reported previously.

BIBLIOGRAPHY

- (1) QUASTEL AND WHEATLEY, *Proc. Roy. Soc. (London)*, Series B, **112**: 60, 1933.
- (2) FUHRMAN AND FIELD, *THIS JOURNAL*, **77**: 392, 1943.
- (3) SOSKIN AND TAUBENHAUS, *THIS JOURNAL*, **78**: 49, 1943.
- (4) KEILIN AND HARTREE, *Proc. Roy. Soc. (London)*, Series B, **122**: 298, 1937.
- (5) PATCH, MORRISON, CIMINERA AND BEYER, *Proc. Fed. Am. Soc. Exp. Biol.* **3**: 35, 1944.
- (6) POTTER AND ELVEHJEM, *J. Biol. Chem.*, **114**: 495, 1936.

LOCAL NERVOUS TISSUE CHANGES FOLLOWING SPINAL ANESTHESIA IN EXPERIMENTAL ANIMALS

CO TUI, M D , A. L. PREISS, M D , I. BARCHAM, M D , AND
MARSHALL I. NEVIN, M D.

With the technical assistance of ARTHUR E. WALLEN

From the Laboratory of Experimental Surgery, New York University College of Medicine

Received for publication July 15, 1943

HISTORICAL REVIEW The histological changes produced by spinal anesthetic agents on the human spinal cord were studied by Spielmeyer (1) in 1908 and by Lindenmulder (2) in 1932. The former author examined the spinal cords of two patients, one dying after stovaine and the other after apothesine spinal anesthesia.

In experimental animals Van Lier (3) in 1907 studied the effects of stovaine-suprarenalin and Wassildo (4) those of stovaine, tropococaine, alpin and procaine. Both of these studies were done on rabbits. Davis, Haven, Given and Emmett (5) in 1931 noted the effects of nupercaine and different procaine combinations (spinoecaine, gravocaine and sculoecaine) on dogs' cords in doses given to patients. These authors also called attention to the fact that local anesthetic agents are hemolytic and therefore, according to Weil (6) also myelolytic agents. Finally the work of Lund, Essex and Kernochan (7), also on dogs, with procaine showed that the concentration of the drugs used was a large factor in the changes in function and in histology of the cord.

Koster and Kasman (9) in 1931 attempted to control quantitatively the concentration of the drug coming in immediate contact with the nervous tissues by injecting 0.21 mgm of procaine per gm weight of autumn frog, dissolving the drug in 0.1 cc of 0.75 NaCl solution and injecting it into the urostyle. This dose was based on the quantitative determination by Bieter, Harvey and Burgess (9) on the spinally effective dose of procaine in frogs.

Thus at least 7 local anesthetic agents have been investigated from the standpoint of their histological effects on the nervous tissues: stovaine, tropococaine, procaine, nupercaine, alpin, apothesine and nupercaine. In these studies, 5 species of animals were used, men, monkeys, dogs, rabbits and frogs. There is uniformity of opinion on the tissue changes in the cord. These are chromolysis, diffuse parenchymatous degeneration, swelling of the cell bodies, dissolution of the Nissl's granules, edema of the nuclear membrane, eccentricity of the nucleus, and inflammatory reaction in the arachnoid with thickening of that membrane. The ganglion cells also participate in these changes. There is, however, divergence of view on the permanence of these changes. Van Lier and Koster and Kasman maintain that these changes are transitory while Spielmeyer and Davis et al find some of them relatively permanent—such as fibrotic scarring of the meninges.

Besides the fact that these two groups of workers were using different experi-

METHODS AND TECHNIQUE 135 animals were used in this study, 81 chinchilla rabbits 54 cats. The concentrations arbitrarily chosen but consistently used for each drug were a) minimum anesthetic concentration (MAC), b) half minimum lethal concentration ($\frac{1}{2}$ MLC) and c) minimum lethal concentration (MLC), using for histological study the animals which survived the injection. The volume injected was in each case 0.02 cc per cm spinal length of the animal. Since the rabbits ranged from 25 to 30 cms in spinal length averaging 27.5 cms, the volumes injected were from 0.5 to 0.6 cc averaging 0.565 cc. The cats ranged between 45 and 49 cm and averaged 48.5 cm spinal length and received from 0.9 to 0.98 cc (average 0.97 cc) of the solution.

The techniques of making the solutions and performing the injections have been described in previous work (11).

Table 1 gives the concentration of each drug used as well as the distribution of the animals in the various groups. It will be noted that there are 18 groups of 3 cats each and 27 groups of 3 rabbits each making a total of 45 groups. The necessity of using phenobarbital as a premedication in all the cats made it impossible to determine the MAC for this species.

TABLE 1

Showing distribution of animals in the different dosage groups with average doses for each group calculated from their spinal lengths of each individual animal

DOSAGE	DRUGS	CONC	CATS						COVC	RABBITS					
			2 da cds		5 da cds		14 da cds			2 da cds		5 da cds		14 da cds	
			No	A V dose	No	A V dose	No	A V dose		No	A V dose	No	A V dose	No	A V dose
		%		mgm		mgm		mgm	%		mgm		mgm		mgm
MAC	Procaine								0.9	3	5.07	3	5.07	3	5.15
	Monocaine								0.5	3	2.83	3	2.75	3	2.87
	Nupercaine								0.3	3	1.8	3	1.53	3	1.72
Half MLC	Procaine	16.5	3	159.6	3	168.3	3	162.525	3	3	17.1	3	17.5	3	36.12
	Monocaine	12.5	3	118.75	3	123.75	3	118.75	6	3	32.53	3	34.5	3	34.2
	Nupercaine	12.5	3	123	3	123.25	3	126.75	1.2	3	6.9	3	7.3	3	7.28
MLC	Procaine	33	3	318.45	3	298.45	3	333.3	6	3	33	3	35.4	3	36.12
	Monocaine	25	3	247.5	3	231.5	3	242.5	12	8	63.6	3	80.4	3	80.4
	Nupercaine	25	3	235	3	243.5	3	258.5	2.4	3	12.12	8	13.3	3	13.8

All animals which did not die recovered from the sensory and motor paralysis within twenty four hours after the spinal injections and none showed any detectable untoward effects later. The animals were killed after two, five, and fourteen days respectively by bleeding. The spinal cord and its coverings were carefully removed from the spinal column and sections corresponding to the site of the injection (lumbosacral segments) were placed in 10% formalin. In removing the cord from the bony canal it was noted that there was an absence of any evidence of infection or hemorrhage.

The sections were stained with 1) hematoxylin Eosin for the general configuration and for evidence of meningeal reaction, 2) cresyl violet for the study of nerve cells and cytoplasmic granules (Nissl bodies) and 3) the Davenport modification of Cajal's silver stain for observation of the collagen fibres, axis cylinders and dendrites.

EXPERIMENTAL RESULTS The histological findings in all the groups were surprisingly uniform and consistent in both species of animals. The presence of the larger amount of spinal fluid in the cat did not, apparently, modify the picture. Since the histological picture of cord changes caused by the other drugs have been reported and since monocaine is the only new anesthetic

agent, only the cord sections of the animals given this latter drug will be given in the illustrations.

M.A.C. CORDS. In the 27 rabbits receiving the M.A.C. of the three drugs, there was no discernible reaction in either the meninges or the cord in any of the sections, whether on the second, the fifth or the fourteenth day.

The myelin sheath stain brought out no evidence of degeneration anywhere in the white matter. The meninges were normal throughout, there being no evidence of inflammatory reaction. The gray matter was likewise normal, the nerve cells being of normal size and shape and the indentations of cellular outlines being plainly visible. The normal cell processes were likewise visible and the Nissl bodies presented no abnormalities. The nucleus was centrally placed showing no evidence of chromatolysis nor could any glia reaction be seen in any of the sections.

HALF M.L.C. CORDS. There were 54 sections under this dosage, 27 of cats and 27 of rabbits. Two day sections of the cords treated with each of the three drugs presented definite evidence of moderately severe pathological changes. The meninges showed inflammatory reaction without the presence of polymorphonuclear leukocytes. Perivascular infiltrations of plasma cells and lymphocytes were marked, extending along the meningeal layers. The white matter showed definite degeneration of the myelin sheaths (fig. 1), the round outlines of which were rarely visible; whatever part of the sheath was present, was distorted. This degeneration was seen throughout the white matter from the periphery to the borders of the grey. In the latter there was a moderately severe glia reaction and the nerve cells especially in the anterior horn presented definite changes. The indentations of the cell outline were lost, the cell outline having become more or less rounded. The Nissl bodies had for the most part lost their granular appearance, many of the nerve cells showing only a smooth homogeneous matrix. The nuclei as a rule were displaced to the periphery and were undergoing chromatolysis.

Five day cord sections presented considerable regression of the above changes. The round cell infiltration of the meninges had mostly cleared; the rounded myelin sheath outlines had reappeared in 60-75% of the white matter and only that portion adjacent to the periphery still remained unaltered from the second day appearance (fig. 2). In the gray matter the nerve cells had recovered considerably, with the normally indented cell outlines returning, the nucleus moving closer to the center and the granular Nissl bodies reappearing.

The fourteenth day specimen presented almost complete return to normal, all the inflammatory meningeal reaction having disappeared. The myelin sheaths had reestablished their rounded contours (fig. 3), the nerve cells in the grey matter had also recovered and only a slight glia reaction still remained.

M.L.C. There were likewise 54 sections under this dosage. Two days sections of the spinal cords subjected to the minimal lethal concentration evidenced the most severe pathological changes of all. There was an intense round cell infiltration of the meninges with associated perivascular cuffing. The white matter (fig. 4) presented considerable degeneration of the rounded myelin



FIG. 1. TWO DAY RABBIT SPINAL CORD SECTION FOLLOWING HALF MLC OF MONOCAINE FORMATE

Davenport modification, Cajal stain. Note moderately severe degeneration and distortion of myelin sheaths in white matter.



FIG. 2 FIVE DAY RABBIT SPINAL CORD SECTION FOLLOWING MLC OF MONOCAINE FORMATE

Davenport modification, Cajal stain. Note considerable recovery and regeneration of myelin sheaths of white matter, as compared with fig. 1.

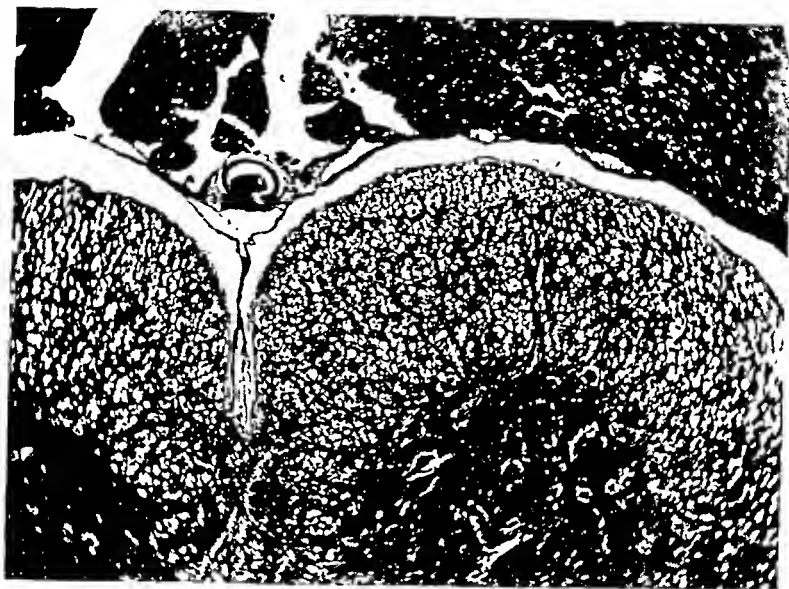


FIG. 3. FOURTEENTH DAY RABBIT SPINAL CORD SECTION FOLLOWING HALF MLC OF MONOCAINE FORMATE

Davenport modification, Cajal stain. Note return of myelin sheaths in white matter completely to normal state as compared with figs. 1 and 2.

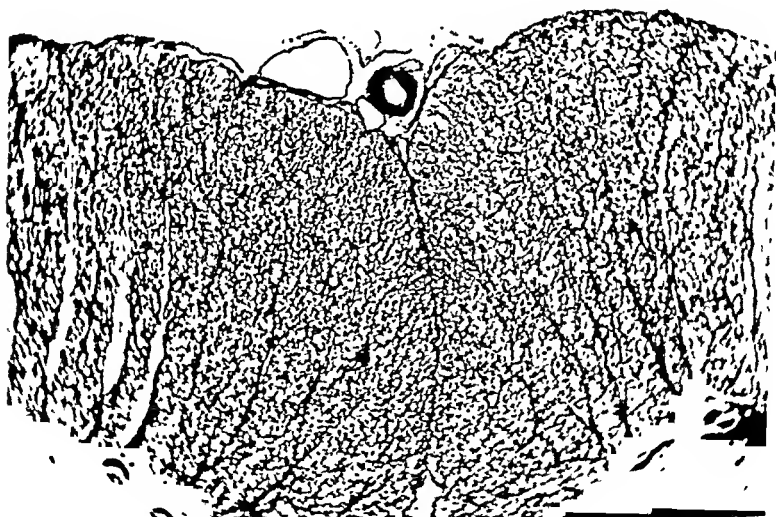


FIG. 4. TWO DAY RABBIT SPINAL CORD SECTION FOLLOWING MLC OF MONOCAINE FORMATE

Davenport modification, Cajal stain. Severe degeneration of myelin sheaths and axon cylinders throughout the white matter. Note greater severity of degenerative process as compared with two day section following half minimal lethal dose in fig. 1.

sheaths for the entire width. In the gray matter the nerve cells were rounded in shape, the Nissl's bodies had disappeared and the nucleus had undergone considerable chromatolysis in its eccentric position at the periphery of the cell. The nerve cells in the anterior horn were particularly affected. There also was a severe glia reaction. Sections from the animals which were killed 5 days after the injection presented little difference from the two day sections except for some slight recession of the infiltration of the meninges (fig 5). Fourteen day sections presented considerable improvement, however, the round cell infiltration of the meninges had almost completely disappeared but fibrotic changes were noted in some places. In the white matter there were about 75-80% regenera-

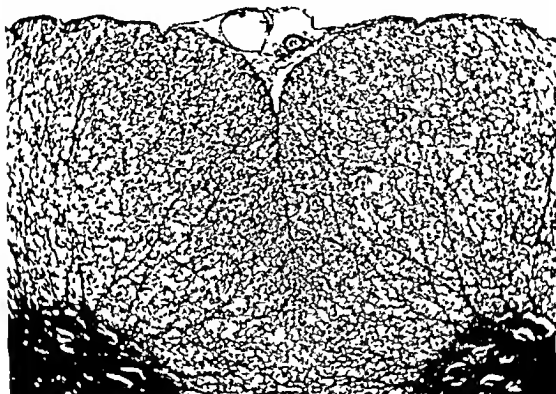


FIG 5 FIVE DAY RABBIT SPINAL CORD SECTION FOLLOWING MLC OF MONOCAINE FORMATE

Davenport modification, Cajal stain. Note similarity of degree of degeneration in the white matter as compared with fig 4.

tion of the myelin sheaths, the greater amount of regeneration having occurred closer to the gray matter (fig 6). In the gray matter the nerve cells had mostly returned to normal but a moderate glia reaction remained.

There was more severe involvement of the anterior horn cells in the animals injected with half the MLD. This may be due to the position of the animals which were strapped to a hammock with the ventral aspect of the body dependent. The more concentrated solutions would therefore tend to stay longer in contact with the anterior than with the posterior aspect of the cord.

COMMENTS. Attention must be called to the fact that by employing the pharmacologic factors of MAC and MLC in this investigation the tissue effects of these drugs are studied from the standpoint of functional effect.

The results of the experiments may be stated briefly. If the intraspinal injections of the M.A.C. of the drugs caused any reactions in the spinal cord of rabbits these reactions were no longer discernible two days after the injection. When the concentration was increased to the half minimum lethal concentration, the tissue changes were fairly marked on the second day, began to show signs of regression on the fifth day and had almost all disappeared on the fourteenth day. With the minimum lethal concentration, however, the tissue changes were more intense, persisting beyond the fifth day but were practically absent on the fourteenth day, leaving some glia reaction.



FIG. 6. FOURTEEN DAY RABBIT SPINAL CORD SECTION FOLLOWING MLC OF MONOCAINE FORMATE

Davenport modification, Cajal stain. Considerable regeneration of myelin sheaths in white matter to be noted as compared with figs. 4 and 5. Nevertheless incompleteness of recovery is to be observed, compared to complete recovery of fourteen day section following half minimal lethal dose as in fig. 3.

Some of the implications of these findings may be set forth:

The tissue effects of procaine, monocaïne formate and nupereaine are of comparable intensity when the drugs are given in comparable functionally active concentrations. This in turn implies a close parallelism between pharmacologic action and tissue reaction. The M.A.C. caused no changes discernible on the two-day sections in any of the three drugs while the half M.L.C. and M.L.C. produced tissue changes almost of parallel intensity for each of the concentrations. If this parallelism is confirmed in the case of other local anesthetic agents it would probably signify that here pharmacologic action and tissue changes are inseparable.

With low concentrations the effects are transitory, thus confirming the work of both Van Lier and of Koster and Kisman but with high concentrations the tissue reaction is more prolonged confirming the work of Spielmeyer and Davis.

There is a basic similarity in the nervous tissue changes produced by these three drugs in rabbits and cats in this work and in the work of others with one of these drugs (procaine) and with other members of the cocaine series in frogs, dogs, monkeys and man. This again indicates a parallelism between pharmacologic effect and tissue response.

In deciding which of these three drugs is to be used as a preferable spinal anesthetic agent, the factor of tissue reaction may be eliminated from consideration. Anesthetic effectiveness and acute toxicity then still remain determining factors.

SUMMARY AND CONCLUSION

1 The tissue changes caused in the spinal cord in the rabbit and cat by the subarachnoidal injection of procaine hydrochloride, monocaine formate and nupercaine in doses quantitated as to their functional effect were studied.

2 The tissue changes if any of M A C were not discernible in two days. With the half M L D the changes reached a maximum on the third day (two day cord) beginning to regress on the sixth day but disappearing almost completely by the fifteenth day.

3 The tissue changes caused by the M L C were more marked than those caused by half M L C and persisted longer showing no regression on the sixth day and still reaction on the fifteenth day.

4 There appears to be a close parallelism between the intensity of pharmacologic action and of tissue changes in all the three drugs studied.

5 The above results seem to reconcile the two schools of thought on the permanence of tissue changes caused by spinal anesthetic agents.

The authors wish to thank the Novocol Chemical Mfg. Co. for making available a supply of monocaine formate for this work.

REFERENCES

- 1 SPIELMEYER W. *Munchen med. Wochenschr.* 55: 1679, 1905.
- 2 LINDENULLDER F. G. *J. Amer. Med. Assoc.* 99: 210, 1932.
- 3 VAN LIER E. H. *Beitr. zur klin. Chir.* 53: 413, 1907.
- 4 WASSILDO F. *Arch. f. klin. Chir.* 85: 1017, 1908.
- 5 DAVIS L., HAVEN H., GIVINS J. H., ENNETT J. J. *Amer. Med. Assoc.* 97: 1781, 1931.
- 6 WILK A. *Arch. f. exper. Path. u. Pharmacol.* 154: 925, 1930.
- 7 LUNDY J. S., ESSIX H. C., KERNACHAN J. W. *J. Amer. Med. Assoc.* 101: 1546, 1933.
- 8 KOSTER H. AND KISMAN L. P. *Ann. Surg.* 25: 277, 1934.
- 9 BIFTER R. N., HARVEY A. M. AND BURGES W. W. *THIS JOURNAL* 45: 291, 1932.
- 10 BIFTER R. N., CUNNINGHAM R. W., LENZ O. AND MCNEARNEY J. J. *THIS JOURNAL* 57: 221, 1936.
- 11 CO TUI F. W. *THIS JOURNAL* 48: 223, 1933.
- 12 CO TUI F. W., PREISS A. J., BLUMSTEIN C. I. AND RUCCIERI W. F. *THIS JOURNAL* 75: 137, 1912.
- 13 ARAMSON D. I. AND GOLDBERG S. D. *THIS JOURNAL* 62: 60, 1938.

A DISTRIBUTION METHOD FOR THE DIFFERENTIATION OF URINARY EXCRETION PRODUCTS OF THE SULFONAMIDE DRUGS AND THE ROLE OF THESE PRODUCTS IN UROLITHIASIS

JOHN V. SCUDI AND VIOLA C. JELINEK

From the Research Laboratories of Merck & Co., Inc.

Received for publication February 11, 1944

Following oral administration, the heterocyclic sulfonamide drugs pass, in part, into the portal circulation and reach the liver. Here, the drugs undergo a series of metabolic reactions which determine to a large extent whether or not the products of these reactions will form uroliths. The products of these reactions, which appear in the urine, may be divided into an *organic-soluble group* and a *water-soluble group*. The former group includes those products which may precipitate in the urinary tract, whereas the water-soluble group includes those products which do not form uroliths. Information concerning these detoxication products is of fundamental interest in the etiology of sulfonamide urolithiasis. In order to determine the proportion of water-soluble and organic-soluble products in the urine, a distribution method was devised, and this method was used to study the urinary elimination of a number of sulfonamides in the rat.

EXPERIMENTAL. Each of six drugs (sulfanilamide, sulfapyridine, sulfathiazole, sulfadiazine, and sulfapyrazine) was given by stomach tube to groups of 3 rats at the uniform dose of 100 mgm. per kg. body weight per day, in the form of a 2 per cent suspension in 10 per cent acacia. The first 48-hour urine sample was collected, and a second 48-hour urine sample including the urinary output after the sixth and seventh doses was also collected. Additional groups of animals were given acacia without the sulfonamide drugs and comparable urine samples were collected for control purposes.

Three series of solutions were prepared for the distribution studies.

1. The 48-hour urine samples were analyzed according to Biatton and Marshall (1);¹ diluted, and adjusted to pH 7.46 at concentrations equivalent to 5.0 mgs. per cent sulfanilamide.

2. Volumes of control urine² equal to those used in the corresponding 48-hour samples were added to slightly alkaline stock solutions of the drugs, and the solutions were adjusted to an equivalent concentration and pH.

¹ Analyses were direct determinations performed without hydrolysis. Since acetylated forms do not diazotize unless hydrolyzed, these are eliminated from consideration, although organic-soluble and water-soluble detoxication products may be acetylated.

² Several of the sulfonamide drugs are known to produce albuminuria (2) and since the sulfonamides are "bound" by protein (3) all urine samples were examined with a heat and acetic acid method. Traces of protein were present in the 48-hour urines, but equal amounts were present in the control urines. Since the protein present in the control urine did not interfere in the determination of the distribution coefficients, it is reasonable to assume that it will not interfere in the extraction of the 48-hour urine. Nevertheless, drug dosage was planned to yield urinary concentrations above 25 mgs. per cent (as sulfanilamide). At these levels the 48-hour urine samples must be diluted at least one to ten before the butanol extraction.

3 Aqueous solutions of the various drugs were adjusted to equivalent concentrations and pH

To 25 cc samples of adjusted solutions 1, 2, and 3, there was added 25 cc of a McIlvaine buffer, at pH 7.46, and 50 cc of reagent *n*-butanol, and the systems, placed on a shaking machine in all-glass containers, were shaken at room temperatures. After equilibration, which is attained within one hour, the volumes of the 2 phases were measured, and 1 cc samples of the butanol and of the aqueous phases were diluted to 100 cc with water and analyzed. The concentration in the butanol phase, C_1 , and the concentration in the aqueous phase, C_2 , were used to calculate the ratio K_1 . After these initial determinations, the pH of the aqueous phase was checked using the glass electrode, and a second extraction was performed with an equal volume of butanol to give C_1^1 , C_2^1 and K_2 . It was not always possible to obtain K_2 values with aqueous solutions of these drugs, for example, with solutions of sulfapyridine at pH 7.46 and with sulfapyrazine at pH 6.0, so much of the drug was extracted by the butanol that insufficient was left in the aqueous phase to give reliable readings in the photoelectric colorimeter.

With sulfadiazine and sulfapyrazine it was found desirable to perform the extractions at pH 6.0. These drugs, which undergo appreciable salt formation at pH 7.46, possess relatively high water solubilities and are not readily differentiated from their more water-soluble metabolites. At pH 6.0, however, a larger portion of the unchanged drug migrates into the butanol phase, and the differentiation is more clearly apparent.

RESULTS Recovery data (columns 1 and 2 of table 1) indicate an analytical variation of ± 2.3 per cent with a maximum deviation from the mean of 5.8 per cent.

The ratios K_1 and K_2 obtained with the aqueous solutions and with the control urine samples are distribution coefficients. These should be constants. Examination of the data (columns 5 and 9) indicates that the widest average deviation from the mean value for any sulfonamide studies is ± 6 per cent and the widest deviation from the mean never exceeds 15 per cent. Thus, changes in concentration in going from K_1 to K_2 , slight changes which may have occurred in the pH, the presence of urine in the aqueous phase, temperature variations, etc., produce variations in the distribution coefficients which fall within these limits. As anticipated, the distribution coefficients of the heterocyclic sulfonamides measured at pH 7.46 parallel the pK values (column 6) reported by Bell and Roblin (4).

The values of K_1 and K_2 obtained with the 48 hour urine sample are not distribution coefficients. They will be called experimental ratios. They are not and should not be constants. In the determination of these experimental ratios a mixture of the unchanged drug and its water soluble metabolites is subjected to butanol extraction. Since the water soluble products will remain essentially in the aqueous phase the ratio of C_1 to C_2 should give values which fall below the distribution coefficient of the given drug. If this is not clearly evident in the initial experimental ratio, K_1 , it should become so in K_2 , because the initial butanol extraction removes much of the unchanged drug leaving a relatively higher concentration, C_2^1 , of water soluble material, and a lower concentration, C_1^1 , of butanol soluble material for the second extraction. Comparison of the experimental ratios and the distribution coefficients (table 1) illustrates these points. For sulfapyridine, sulfathiazole, and sulfamerazine, the K_1 experimental

TABLE 1
The distribution of urinary sulfonamides between water and butanol

DRUG AND pH OF EXPERIMENT	RECOVERY DATA		C_1 BUTA- NOL	C_2 H ₂ O	K_1	pK	C_1' BUTA- NOL	C_2' H ₂ O	K_2	% WATER SOLUBLE METABOLITES	
	Conc. present	Conc. found								C_1	C_2
Sulfanilamide H ₂ O	4.90	4.82	2.80 2.70	2.10 2.00	1.30 1.35	10.43	1.25 1.13	1.00 0.80	1.30 1.40		
Control urine			2.80	2.00	1.40		1.15	0.90	1.28		
pH 7.46 Test urine			2.75 3.00	2.30 2.40	1.20 1.25		1.25 1.20	1.50 1.30	0.83 0.92		12 9
Sulfapyridine H ₂ O	5.00 4.80	4.88 4.80	3.70 3.75	1.00 0.95	3.70 3.95	8.43					
Control urine			3.90 4.10	1.10 1.20	3.55 3.42						
pH 7.46 Test urine		4.92	2.70	2.40	1.12		0.80	2.25	0.35	35	43
	4.70 5.20	5.24	2.60	3.10	0.84		0.75	2.75	0.27	46	45
Sulfathiazole H ₂ O	5.00	5.10	3.60 3.50	1.40 1.20	2.57 2.91	7.12	1.05 0.80	0.04 0.30	2.62 2.67		
Control urine			3.60	1.30	2.77						
pH 7.46 Test urine			3.20 3.20	1.60 1.80	2.00 1.78		1.05 1.00	1.00 1.11	1.05 0.91	9 13	13
	4.80	4.76									
Sulfamerizine H ₂ O			3.00 3.20	2.10 1.80	1.43 1.78	7.06	1.35 1.10	0.85 0.63	1.59 1.76		
Control urine			3.00	2.10	1.43		1.20	0.85	1.41		
pH 7.46 Test urine			2.50 2.70	2.40 2.30	1.04 1.17		1.20 1.10	1.60 1.38	0.75 0.80	21 14	20 17
	4.50	4.76									
Sulfadiazine H ₂ O	5.00	4.96	1.80 1.80	3.50 3.20	0.51 0.56	6.48	1.30	2.15	0.60		
Control urine....			1.70	3.60	0.47		1.25	2.60	0.48		
pH 7.46 Test urine			1.60 2.00	3.50 3.60	0.46 0.56		1.35 1.33	2.70 2.70	0.50 0.49		
	4.70	4.74									

Concentrations are expressed in mgs. per cent in terms of Sulfanilamide.

* Using the values of C_1 and C_1' found with the test urine, and average values of the distribution coefficients, the concentrations C_2 and C_2' were calculated from $K = C_1/C_2$. The calculated C_2 values were subtracted from the C_2 values found to give the concentration of the water-soluble metabolites. This concentration is expressed as the percentage of the original concentration recorded in column 1.

TABLE 1—Continued

DRUG AND pH OF EXPERIMENT	RECOVERY DATA		C_1 BUTANOL	C_2 H ₂ O	K_1	K_2	C_1 BUTANOL	C_2 H ₂ O	K_1	% WATER SOLUBLE METABOLITES	
	Conc present	Conc found								C_1	C_2
Sulfadiazine H ₂ O	5 10	5 08	3 75	1 25	3 00		0 88	0 30	2 93		
Control urine	5 10	5 20	3 80	1 35	2 82		1 00	0 36	2 78		
pH 6.0 Test urine	5 50	5 46	3 80	1 63	2 33		1 00	0 60	1 67	6	4 5
Sulfapyrazine H ₂ O	4 80	4 86	3 90	0 80	4 88	6 04					
Control urine	5 20	5 28	4 20	0 95	4 42	/					
pH 6.0 Test urine	5 50	5 32	4 10	1 20	3 42		0 80	0 40	2 00	6 0	4 2

ratios are smaller than the corresponding distribution coefficients, and the K_2 ratios are smaller still. For sulfanilamide, the K_1 ratio is not appreciably smaller than the distribution ratio, but the K_2 ratio is significantly smaller. Thus, the determination of the experimental ratio K_2 may disclose the presence of water soluble products not detected in the K_1 determination.

If one assumes that only the unchanged drug migrates into the butanol phase, the concentration of the water soluble products in the 48 hour urine may be calculated from $K = C_1/C_2$. Knowing the distribution coefficient, K , and C_1 , the concentration of the unchanged drug found in the butanol phase, its concentration in the aqueous phase C_2 , may be calculated. C_2 , experimentally determined, minus C_2 , calculated, is essentially equal to the concentration of the water soluble excretion products. Values so calculated are listed in column 10, of table 1 as percentages of the diazotizable materials appearing in the 48 hour urine samples. Approximately 40 per cent of the so called 'free' sulfapyridine in rat urine appears in a water soluble form. This is in agreement with the value of 40 per cent estimated by us (5) by other means. About 10 per cent of the urinary sulfanilamide appears in a water soluble form. Compared to this value, it may be noted that Shelswell and Williams (6) estimated by other means that 6 to 12 per cent of the sulfanilamide was eliminated as an ethereal sulfate in the rabbit.

The assumption that only the unchanged sulfonamides will migrate into the butanol phase is not entirely justified. Slightly modified forms of the drugs, and small amounts of the water soluble products will be extracted by the butanol. Any partial hydrolysis of an acetyl derivative which may have occurred during the test procedure would liberate more of the unchanged drug and partial hydrolysis of an ethereal sulfate or glucuronide linkage would presumably liberate a hydroxysulfonamide. These products would also be extracted by the butanol. It should be pointed out, however, that these effects will diminish the apparent concentration of the water soluble excretion products. Hence these factors in no way invalidate the findings presented.

It is interesting to note that the percentage of the heterocyclic sulfonamides which appears in a water-soluble form parallels the pK values reported by Bell and Roblin (4). This parallelism suggests that the heterocyclic sulfonamides possessing the lowest pK values are, in this respect, the least metabolized. It might appear that these drugs, circulating largely in the form of soluble anions in body fluids, are readily eliminated and, thus, escape metabolism. In this connection, it may be noted that sulfanilamide, although a very weak acid, is relatively soluble, and it, too, is converted but slightly to water-soluble excretion products. Sulfamerizine is more soluble than sulfathiazole at pH 7.4; yet, it is metabolized to a slightly greater extent. Since sulfamerizine is retained in the animal organism for comparatively long periods of time (7), this retention, related possibly to protein binding, tubular reabsorption, etc., may influence the metabolism of this drug. Although other factors are involved, the relationship of the degree of this metabolism to the solubility and to the tendency of the drug to dissociate³ appears to be significant.

DISCUSSION. The urinary excretion products of the sulfonamide drugs have been divided, for quantitative study, into two groups; namely, an organic-soluble, and a water-soluble group. The former includes those relatively insoluble products which may precipitate within the urinary tract, whereas the latter group includes those comparatively soluble products which do not form uroliths.

The organic-soluble group consists essentially of the parent drug (8), an N_4 -acetyl derivative (8, 9, 10), a monohydroxyl derivative (11), and possibly other, as yet uncharacterized, products.⁴ Each of the three known types of product has been found in urinary concretions. Stones composed of unchanged sulfadiazine and of unchanged sulfamerizine have been reported (7). Uroliths composed almost entirely of N_4 -acetylsulfapyridine were the very first uroliths to be reported in the literature (2), and concretions consisting of an almost pure hydroxysulfonamide were observed in monkeys (12).

The water-soluble group of products measured by means of the distribution method, consists essentially of glucuronides or ethereal sulfates of monohydroxy derivatives of the sulfonamide drugs. That such urinary products exist has been clearly demonstrated by the isolation of products from urine; for example, a hydroxysulfapyridine glucuronide has been isolated from dog urine (11), and Thorpe and Williams announced the isolation of glucuronides of a hydroxysulfathiazole and of a hydroxysulfanilamide (13). Apparently no metabolic products of sulfamerizine, sulfadiazine or sulfapyrazine have yet been isolated. Without their isolation and identification it is not possible to draw absolute conclusions

³ It may be recalled at this point that the sulfonamide drugs are more soluble in alkaline than in neutral solution because the drugs form relatively soluble salts. This increased solubility is governed by the tendency of the drugs to undergo anionic dissociation, but the terms "solubility" and "dissociation" are not interchangeable. Different drugs possessing the same pK constants may well possess different solubilities, and further, the physiological behavior of the ion differs from that of the undissociated drug.

⁴ For purposes of simplicity only free forms of the drugs—i.e., forms which possess free, diazotizable arylamine groups—were considered in the distribution method described above.

regarding their nature, but it is reasonable to assume that these drugs are metabolized in much the same manner as the three preceding sulfonamide drugs. As noted above, products of the water soluble group do not form uroliths. Hence, each portion of a drug so excreted, reduces the amount of the drug available for urolith formation. Conversely, any interruption of the chain of metabolic reactions which yield these products increases the amount of the drug to be excreted in some other form. That interruption of this chain of reactions may lead to an increased incidence of urolithiasis was demonstrated by chloroform and phosphorus liver damage in the rat (5). Graded damage of the liver reduced the urinary output of hydroxysulfapyridine glucuronide, and the incidence of uroliths was increased from 10 to 25 to 60 per cent. More recently, an increased incidence of renal complications was observed in the course of sulfonamide therapy among patients suffering of liver disease (14).

SUMMARY

The urinary excretion products of a number of sulfonamide drugs have been divided, by means of a distribution method, into a so-called *organic soluble* group and a *water soluble* group. The products within each group have been considered and their relationship to the incidence of sulfonamide urolithiasis has been discussed.

Of the "free" sulfonamide (i.e., not including acetyl derivatives) found in the urine, it has been estimated that 40 per cent of the sulfapyridine, 10 to 20 per cent of the sulfanilamide, sulfathiazole and sulfamerazine, and 4 to 6 per cent of the sulfadiazine and sulfapyrazine exists in the form of water soluble metabolites. The diminution of these values parallels the pK values of the heterocyclic sulfonamides.

BIBLIOGRAPHY

- 1 BRATTON, A. C., AND MARSHALL, E. K., JR., *J. Biol. Chem.*, **128**, 537, 1939.
- 2 ANTOPOL, W., AND ROBINSON, H., *Proc. Soc. Exper. Biol. Med.*, **40**, 428, 1939.
- 3 DAVIS, B. D., AND WOOD, W. B., *Proc. Soc. Exper. Biol. Med.*, **51**, 283, 1942.
- 4 BELL, P. H., AND ROBLIN, R. O., JR., *J. A. C. S.*, **64**, 2905, 1942.
- 5 SCUDI, J. V., AND ROBINSON, H. J., *Amer. J. Med. Sci.*, **201**, 711, 1941.
- 6 SHELSWELL, J., AND WILLIAMS, R. T., *Biochem. J.*, **34**, 528, 1940.
- 7 WELCH, A. D., MATTIS, P. A., LATVEN, A. R., BENSON, W. M., AND SHIELS, E. H., *J. Pharmacol.*, **77**, 357, 1943.
- 8 MARSHALL, E. K., JR., BRATTON, A. C., AND LITCHFIELD, J. T., JR., *Science*, **88**, 597, 1938.
- 9 MARSHALL, E. K., JR., EMERSON, K., JR., AND CUTTING, W. C., *J. Amer. Med. Assoc.*, **108**, 953, 1937.
- 10 RATISH, H. D., BULLOWA, J. G. M., AMES, J. B., AND SCUDI, J. V., *J. Biol. Chem.*, **128**, 279, 1939.
- 11 SCUDI, J. V., *Science*, **91**, 486, 1940; *Proc. Soc. Exper. Biol. Med.*, **55**, 197, 1944.
- 12 Unpublished data to be reported from these laboratories.
- 13 THORPE, W. V., AND WILLIAMS, R. T., *Nature*, **145**, 686, 1940.
- 14 PETERSON, O. L., DEUTSCH, E., AND FINLAND, M., *Arch. Intern. Med.*, **72**, 591, 1943.

THE TOXICITY AND TRYPANOCIDAL ACTIVITY OF SOME ORGANIC ANTIMONIALS

L. G. GOODWIN

From the Wellcome Bureau of Scientific Research, 183, Euston Road, London, N. W. 1

Received for publication March 2, 1944

INTRODUCTION. Systematic studies of the biological properties of organic antimonials have been in progress in these laboratories during the last four years, and an account of the excretion of antimony has already been published (Goodwin and Page (1, 2)). The aim of the present work has been to determine and to record in one place the toxicity, irritancy and trypanocidal activity of a series of the better known antimony compounds, and to ensure that this information shall have been obtained by recent and reliable experimental methods. Existing information leaves much to be desired, since there is lack of uniformity in technique and no indication of the variance of results.

MATERIALS. (a) *Tartar emetic and trivalent analogues.* 1. Tartar emetic—potassium antimony^{III} tartrate: (a) Stock crystalline sample, (b) Methyl alcohol precipitated sample.

2. Sodium antimony^{III} tartrate: (a) Stock sample (powder), (b) Methyl alcohol precipitated sample.

3. "Anthiomaline" (May and Baker)—lithium antimony^{III} thiomalate.

4. Stibophen B. P.—sodium antimony^{III} bis-pyrocatechol 3:5-disulphonate ("Fousadin", Bayer).

5. "Stibsol"—sodium antimony^{III}-3-catechol thiosalicylate.

6. Sodium antimony^{III} gluconate.

(b) *Quinquevalent analogues of tartar emetic.* 7. (a) "Solustibosan" (Bayer)—sodium antimony^V gluconate (?), (b) Sodium antimony^V gluconate.

8. Sodium mannitol antimoniate.

9. Sodium antimony^V tartrate.

10. Stibophen containing Sb^V instead of Sb^{III} ("quinquevalent stibophen").

(c) *Quinquevalent compounds that are derivatives of phenylstibonic acid.* 11. "Neostam" (Wellcome Foundation)—stibamine glucoside.

12. "Neostibosan" (Bayer)—polymerised diethylamine *p*-aminophenylstibonate.

13. "Ureastibamine" (Brahmachari)—mixture of urcides of *p*-aminophenyl stibonic acid.

14. "Stibacstin"—sodium *p*-acetylaminophenylstibonate.

For the preparation of samples 1(b), 2(b), 5, 6, 7(b), 8, 9 and 10, and for much valuable advice and co-operation in this work, the author is indebted to Mr. W. Solomon, M.Sc., of the Chemical Department, Wellcome Bureau of Scientific Research.

Sodium antimony^{III} gluconate, sodium antimony^V tartrate and quinquevalent stibophen were included for the investigation of the effect of the valency of antimony upon pharmacological properties. All the materials except potassium antimony^{III} tartrate and stibophen were amorphous solids. Quinquevalent stibophen prepared by the oxidation of B. P. stibophen with hydrogen peroxide was found to be a very hygroscopic substance and calculations of dosage used were made from the assayed antimony content of solutions, assuming the formula of the solid to be $C_{12}H_4O_{17}S_4Na_3Sb_2 \cdot 7H_2O$. Stibsol was prepared according to Brown and Austin (3). Samples 1(b) and 2(b) were prepared by heating potassium or sodium hydrogen tartrate with a slight excess of antimony trioxide, and filtration and precipitation of the product with methyl alcohol. Samples 6, 7(b), 8 and 9 were prepared

by the action of antimony tri- or pentachloride upon the appropriate organic acid or alcohol, neutralisation and precipitation with methyl alcohol (4)

METHODS *Toxicity* Mice weighing 13-19 gm or 20-25 gm were used for the toxicity tests. Evidence was obtained from preliminary trials that, as with arsenic compounds, small mice were somewhat more resistant to antimony poisoning than larger ones. Dosage was therefore given in accordance with the suggestion of Durham, Gaddum and Marchal (5), 13-19 gm mice being treated as if they were all of equal weight, and 20-25 gm mice being dosed in proportion to body weight. Injections were made intravenously using wherever possible a constant volume of 0.5 ml of a freshly prepared solution in sterile water per 20 gm mouse. In the case of stibophen and sodium antimony^v gluconate, the auto-claved solutions prepared for human therapeutics were used. Mortality was observed for at least 3 days and the L D₅₀ and fiducial limits were calculated by the method of Bliss (6).

Irritant activity The irritant properties of the substances tested were determined by the method of Paget, Trevan and Attwood (7). Solutions of graded concentration were prepared in distilled water and quantities of 0.05 ml injected intracutaneously into the shaven flank of a guinea pig. The least concentration which produced a clear local reaction during an observation period of 3 days was recorded, (minimal necrosing concentration). Each substance was tested on at least 2 guinea pigs.

Trypanocidal activity A suspension of *Trypanosoma equiperdum* in citrate glucos-saline, (sodium citrate 0.5%, glucose 1% sodium chloride 0.85%) containing 4,000 organisms per cu mm was prepared from the heart blood of an infected mouse. A series of mice were inoculated intraperitoneally with the suspension, each mouse receiving 0.5 ml (2×10^6 trypanosomes). The following day a drop of the peripheral blood of each mouse was examined microscopically under a cover slip and the small number of mice which did not show parasitaemia during the examination of a few microscope fields viewed with the $\frac{1}{2}$ " objective were discarded. The infected animals were divided into groups and doses of drugs injected subcutaneously, 2 or 3 dose levels differing by the constant factor of $\sqrt{2}$ usually being given. One group was kept as a control and did not receive any dose. The peripheral blood of each mouse was re-examined 24 hours later and the proportion of mice in each group which were 'cleared' of trypanosomes determined. The median effective dose (R D₅₀) and the parameters of the dose-effect curve were calculated directly from the logarithms of the doses and the probits of the percentage responses by Gaddum's method (8).

After the second blood examination, the mice were set aside and the number of survivors in each group counted daily for a further 5 days. The "expectation of life", limited to a possible maximum of 6 days after injection of the drug was then calculated for each group. [For a group of l_0 mice and an experiment of length n days expectation of life is given by $\frac{1}{l_0} \left\{ \frac{1}{2} l_0 + l_1 + l_2 + \dots + l_{n-1} + \frac{1}{2} l_n \right\}$. In the event of all the mice in a group dying before the end of the test, the expectation of life is identical with the mean of the survival times of the individual mice.]

The expectation of life of the group of infected control mice was similarly determined, the value usually being 2-3 days (i.e. 3-4 days after infection). At the end of the test, the expectations of life in days were plotted against the logarithms of the doses and a line fitted graphically to the steepest part of the curve. The positions of maximum response (6 days) and zero response (expectation of life of the control group) were marked on the graph and the dose corresponding to an expectation of life midway between these two extremes was recorded as the 'standard effective dose' (S E D).

Notes upon the trypanocidal test The method described above was designed so that two recent methods of calculation could be applied to the results and so compared. The first part of the test which determines the percentage of mice cleared of trypanosomes in the peripheral blood is almost identical with the method devised by Hawking for the assay of suramin (9) and of organic arsenicals (10). An interval of 24 hours between dosing and ex-

amination for clearance was used in the present work as this period corresponded with the maximum response obtained from a given dose. When left for 3 days as in Hawking's method, some mice had already relapsed, especially with low doses of tervalent compounds.

The second part of the test is based upon the principle of a method described by Bülbring and Burn (11), for the assay of arsenic and antimony compounds, and the results of the present work give some idea of the errors to be expected from a test of this kind. In the original method the daily numbers of survivors in a group were totalled at the end of the experiment and the resulting figure was taken as a measure of the protection afforded by the dose. The calculation of expectations of life is essentially the same process and the results obtained thereby do not differ greatly from those calculated from the totals. If all the mice die before the end of the experiment on the 6th day, both the expectations of life and the Bülbring and Burn totals are estimates of the mean of the survival times of the individual mice in a group. Expectations of life give values more closely approximating to the true mean survival time than do the Bülbring and Burn totals. In such a case the experiment takes the form of a true graded response, limited in accuracy by the length of the intervals at which deaths are observed. If survivors are counted at intervals of 12 hours instead of 24, the test gains in accuracy but is rather less convenient in use. When, as is generally the case with the higher dose levels, some animals are alive at the end of the test, their survival times are arbitrarily taken as 6 days, and the response loses its truly graded character. Longer observation periods than 6 days are inconvenient, for they are time-consuming, there is a greater danger of intercurrent infections and deaths from causes other than trypanosomiasis, and there is still the difficulty of dealing with a few mice which do not relapse (12). The arbitrary completion of the test in 6 days has the effect of "heaping up" the distribution of survival times at that point but this is not serious if the dose levels are carefully chosen. When expectations of life are plotted against logarithms of the doses, the points fall upon a sigmoid curve, and the steepest part of this curve corresponds with an expectation of life midway between the zero and maximum possible responses (fig. 1 (b)). The dose producing this response (the S.E.D.) has been taken in this work as the best measure of the activity of a particular drug for comparative purposes. In cases in which the dose-response curves are parallel it is possible to compare the activities of two substances by Irwin's "method (c)" (13), the expectations of life being treated as true graded responses. Only the values of the slopes of the curves and the standard effective doses are recorded in this paper, however, as there are qualitative as well as quantitative differences in activity between the various substances tested. The results given by Irwin's "method (c)" are not very different from the ratios of standard effective doses.

RESULTS. Toxicity. The L.D. 50 and its limits, and the slope of the regression line fitted to the observations upon the toxicity of each compound are listed in table 1. The results show that the quinquevalent compounds are on the whole less toxic than the tervalent compounds (*cf.* Brunner (14); Bock (15)), and this is particularly evident upon comparison of the toxicities of sodium antimony^{III} tartrate and gluconate and stibophen with the toxicities of the corresponding quinquevalent compounds.

The L.D. 50 values found for potassium and sodium antimony^{III} tartrates are considerably greater than those reported by Fargher and Gray (16) for these compounds, and tests upon the stock laboratory samples 1(a) and 2(a) showed the potassium salt to be less toxic than the sodium salt. Experience with sodium antimony^V gluconate (see below) had shown that the toxicity of this substance could be varied by varying the method of preparation, and as the histories of samples 1(a) and 2(a) were unknown, comparable samples 1(b) and 2(b) were prepared using a method of alcohol precipitation. The resulting materials were

found to have higher antimony contents when assayed by the B P method. The total antimony contents (see table 5) were slightly higher still, showing the presence of a small amount of the quinquivalent form. Samples 1(b) and 2(b) were tested for toxicity on the same day, and were also found to be less toxic than Fargher and Gray's samples, though the potassium salt was this time more toxic than the sodium salt. The varying toxicity shown by samples of potassium antimony^{III} tartrate may explain the divergence of the observations of Rogers (17) and Fargher and Gray (16) who found the potassium salt to be more toxic than the sodium, and of Brahmachari (18) who found the two substances to be

TABLE 1

The toxicity of some organic antimony compounds injected intravenously into mice

SUBSTANCE	TOTAL NO OF MICE USED	b	s	L.D. 50 mg / 0 gm	LD LIMITS (P = 0.95)
1 Tartar emetic (a)	120	7.44	0.41	1.53	91-110
(b)	80	6.59	0.55	0.93	89-112
2 Sodium antimony ^{III} tartrate (a)	60	9.70	0.84	1.14	90-111
(b)	100	8.28	0.59	1.15	92-109
3 Anthiomaline	90	7.12	1.37	3.62	88-113
4 Stibopben	80	3.83	0.61	31.2	81-123
5 Stibsol	80	7.40	1.66	1.11	89-111
6 Sodium antimony ^{III} gluconate	70	6.81	1.41	3.44	89-113
7 (a) Solustibosan	46	18.0	5.09	32.5	04-106
(b) Sodium antimony ^V gluconate	80	0.78	2.20	33.0	93-108
8 Sodium mannitol antimoniate	70	13.0	2.57	102.2	03-107
9 Tartar emetic (Sb ^V)	60	21.5	4.34	5.14	96-104
10 Stibophen (Sb ^V)	70	6.38	1.22	66.6	88-113
11 Neostam	70	4.09	0.83	29.5	82-122
12 Neostibosan	90	6.50	1.03	9.44	89-112
13 Ureastibamine	120	1.29	0.16	4.26	64-157
14 Stibacetin	65	3.68	1.17	5.65	82-122

All mice weighed 20-25 g except those for substances 4, 10, 11 and 12, these weighed 13-19 g.

equally toxic. Fargher and Gray used 'sterilised' solutions for their toxicity tests and in case the higher toxicities observed by them might be due to this, a solution of potassium antimony^{III} tartrate 1(b) which had been autoclaved was compared with a fresh solution. The heated solution was slightly less toxic than the unheated one, so that the high toxicities reported by Fargher and Gray remain unexplained. A slight decrease in toxicity upon autoclaving was also noticed with sodium antimony^V tartrate and gluconate.

The other I.D. 50 values show general agreement with the variously expressed toxicities reported by previous workers. The results for ureastibamine and stibacetin do not differ greatly from those given by Gray, Trevan, Bunbridge and

Attwood (19), though the sample of neostam tested was somewhat less toxic than theirs. The figure for ureastibamine also agrees with the findings of Napier (20) and of Guha, Dutta and Mukerji (21) but the slope of the regression line was so shallow that the result has a large variance. The toxicity of the quinquevalent mannitol derivative is of the same order as the figure quoted by Chung and Chow (22) who state that "white mice can withstand intravenous injections of 0.3 ml. of a 50 per cent solution." This compound had the lowest toxicity of any so far tested. The value given for neostibosan agrees with the result of an experiment on a small number of mice recorded by Napier (23). Stibsol, which has been recommended by Brown and Austin (24) for the treatment of filarial infections in dogs, is very similar to tartar emetic in properties. Solustibosan has a toxicity identical with that of the sample of sodium antimony^v glueonate recorded in table 1, and this value is similar to the findings of Weese (25). However, it was found possible to prepare samples of the glueonate, which although almost equal in antimony content, had L.D. 50 values ranging from 19 to 76 mg./20 gm. This variation appeared to depend firstly upon a factor intrinsic to the method of preparation of the compound, and secondly upon the pH of the injected autoclaved solution. Within the pH range of stability of the substance, acid solutions were very much less toxic than neutral or alkaline solutions.

It is noticeable that the slopes of the regression lines for the quinquevalent analogues of tartar emetic are much steeper than those for the tervalent or the phenylstibonic acid derivatives. Also, with the first type of substance, all deaths observed upon intravenous injection took place within a few minutes, whereas with the other compounds, death was often delayed for 2 or 3 days or longer. Experiments upon anaesthetized cats and rabbits showed that the acute toxic action of the quinquevalent analogues of tartar emetic was upon the heart, and very similar to the effect observed with phenyl stibonic acid derivatives by Chopra (26). The delayed toxic activity of the tervalent compounds is due to degenerative changes produced in the liver and kidneys (27) (18).

Irritant activity. Table 2 shows that the differences between the irritant activities of the quinquevalent analogues of tartar emetic and the rest are even more marked than the toxicity differences. Sodium antimonyⁱⁱⁱ tartrate was slightly, but definitely less irritant than the potassium salt.

Trypanocidal activity. Table 3 and fig. 1 comprise a sample protocol for a complete experiment with tartar emetic. Doses were given in this case to cover the whole of the dose-response curve. Table 4 shows the results of the two methods of assessment of trypanocidal activity. The phenyl stibonic acid derivatives were less active than the tervalent compounds, and neostibosan and the quinquevalent analogues of tartar emetic were devoid of all activity in single doses. Comparison of the two methods of calculation of activity showed that for the tervalent compounds, the R.D. 50 and the S.E.D. were almost identical. With the phenyl stibonic acid derivatives however, the S.E.D. was a little lower than the R.D. 50. A careful study of the day to day degree of infection of the mice treated with these compounds showed that the difference was due not to over-estimation of the R.D. 50 by premature examination for clearance of the blood,

but to the less rapid multiplication of the trypanosomes and the consequent prolongation of survival time. Fig 2 shows a comparison between the development of infection in groups of mice which had the same percentage clearance 24 hours after doses of tartar emetic and stibacetin respectively. Whereas the infection in the mice treated with tartar emetic flared up immediately after the first restraint, the infection in the stibacetin treated animals increased only slowly. These observations are in accordance with the statement of Uhlenhuth, Kuhn and Schmidt (28) that the phenyl stibonic acid derivatives take 2 or 3 days to exert their action although tartar emetic is effective in a few hours. The reason for this difference has been assumed (by analogy with arsenic compounds),

TABLE 2

The irritant activity of organic antimonials upon intracutaneous injection in the guinea pig

SUBSTANCES	REACTION UPON INJECTION (% CONCENTRATION OF SOLUTION)											MINIMAL NECROSING CONCENTRATION %
	100	50	25	12.5	5	2	1	0.5	0.2	0.1	0.05	
1 Tartar emetic							++	+	+	±	0	0.1
2 Sodium antimony ^{III} tartrate							++	++	+	0	0	0.2
3 Anthiomaline					+	+	±	0				1.0
4 Stibophen		++	+	+	0							12.5
5 Stibsol							++	+	+	+	0	0.1
6 Sodium antimony ^{III} gluconate					+++	++	+	+	±	0	0	0.2
7 (a) Solustibosan (b) Sodium antimony ^V gluconate	+	+	+	±	0							12.5
8 Sodium mannitol antimoniate	0	0	0	0	0							>100
9 Sodium antimony ^V tartrate	+	+	+	+	0	0						12.5
10 Stibophen (Sb ^V)		0	0	0	0							>50
11 Neostam					+++	++	+	±	0			0.5
12 Neostibosan						++	+	+	0			0.5
13 Ureastibamine						++	+	+	+	0	0	0.2
14 Stibacetin						+	±	0	0			1.0

to be that quinquivalent antimony must be reduced by the tissues to the trivalent form before it can exert any trypanocidal action. Evidence presented in a previous paper (1) proves that reduction can take place in the body.

In table 5 all the results are summarised, expressed as quantities of metallic antimony. The initial rates of excretion of the compounds after intravenous injection into mice of quantities equivalent to 3-4 mg. of antimony per kg. are also recorded. Most of these results are taken from a previous paper (1), and the rest have been determined by the same technique.

DISCUSSION Reference to table 5 shows that toxicity, irritancy and trypanocidal activity are related to one another but are independent of antimony content. There is some correlation between the above properties and the initial rates of excretion of the compounds, the more rapidly excreted substances being,

as would be expected, less toxic and less active than the others. The differences in rates of excretion are not great enough to account for all the observed varia-

TABLE 3

Typical protocol showing the method of assessment of the trypanocidal activity of tartar emetic by two methods

HAWKING				BÜLBRING & BURN						
Dose	No. of mice	% cleared in 24 hr.	Probit % cleared	No. of survivors (days after dose)						Expectation of life
mg./20 gm.				1	2	3	4	5	6	days
0.0500	10	0		10	10	3	0			2.8
0.0707	20	0		20	20	13	1	0		3.2
0.1000	20	20	4.158	20	20	15	6	2	2	3.7
0.1414	20	65	5.385	20	20	20	18	15	12	5.5
0.2000	20	95	6.645	20	20	20	20	19	16	5.9
Controls.....	20	0		20	20	5	1	1	0	2.9

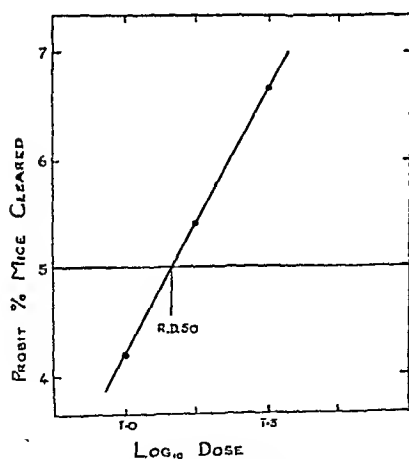
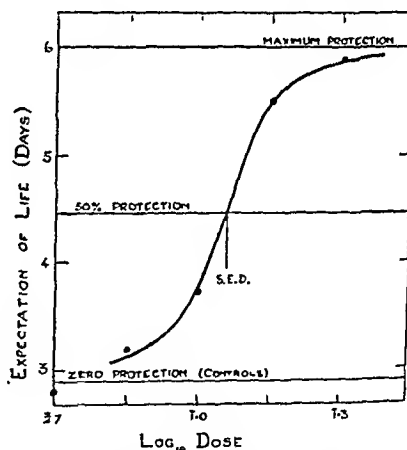


FIG. 1 (a) FITTING REGRESSION LINE

$S(Bn) = 28.760$; $\bar{x} = 1.0936$; $\bar{y} = 4.9320$;
 $b \pm \sigma_b = 8.13 \pm 1.47$; $y - 4.932 = 8.13$
 $(x - 1.0936)$. $R.D. 50 = 0.126 \text{ mg./20 gm.}$

FIG. 1 (b) SLOPE OF STEEP PART OF CURVE
 $b = 11.8$

$S.E.D. > 0.113 \text{ mg./20 gm.}$

tions, however, and there are certainly other factors which determine toxicity. This is evident from the variation in toxicity of samples of sodium antimony gluconate.

The comparison of the results of the Hawking and the Bülbring and Burn

TABLE 4

The trypanocidal activity of some organic antimony compounds when injected subcutaneously into mice infected with *Trypanosoma equiperdum*

SUBSTANCE	EXPT	RAWKING'S METHOD						BULBRING AND BURN'S METHOD					
		No of mice used	b	%	Weighted mean b	R D 50 (mg /20 gm)	Weighted mean R D 50	No of mice used	b	Weighted mean b	S E D (mg /20 gm)	Weighted mean S E D	
1 Tartar emetic	1	20	8.4	3.21	5.44	0.103	0.133			9.11		0.138	
	2				±0.81		±0.003	39	3.5	±1.23	0.163	±0.008	
	3							26	9.3		0.121		
	4	28	3.7	2.23		0.175		40	5.6		0.152		
	5	50	5.1	1.47		0.126		110	7.6		0.113		
	6	58	21.1	2.74		0.125		71	7.7		0.106		
	7	15	2.3	3.90		0.145							
	8	48	10.5	2.43		0.156		63	11.3		0.159		
	9	49	6.7	1.93		0.125		59	7.3		0.157		
	10	28	8.4	2.72		0.116		37	7.6		0.152		
2 Sodium anti mony ^{III} tartrate	1	19	13.6	4.74	8.12	0.115	0.143	20	10.8	0.40	0.100	0.121	
	2	27	8.0	2.40	±1.22	0.161	±0.009	20	9.3	±0.07	0.134	±0.006	
	3	30	6.3	2.64		0.146		30	7.3		0.115		
	4	30	8.7	2.92		0.156		30	7.6		0.125		
	6	27	6.1	2.36		0.127		30	12.4		0.126		
3 Anthiomaline	1				6.92	0.333	0.330	36	7.0	6.29	0.296	0.308	
	2	30	6.6	2.06	±0.99	0.336	±0.023			±0.68		±0.038	
	3	20	16.9	5.03		0.336		40	5.6		0.417		
	4	69	5.1	1.99		0.260		79	4.8		0.230		
	6	59	0.8	2.11		0.326		72	7.5		0.266		
	6	42	5.0	1.06		0.415		57	6.3		0.400		
4 Stibophen	1	40	5.8	1.76	6.81	0.915	0.709	50	8.6	6.54	0.708	0.638	
	2				±1.16		±0.039	45	6.6	±0.66	0.624	±0.036	
	3							89	4.4		0.670		
	4							47	7.0		0.708		
	5							23	6.4		0.653		
	6	30	10.3	3.20		0.686		40	7.5		0.640		
	7	20	11.2	4.25		0.595		29	10.1		0.644		
	8	60	6.4	2.32		0.595		30	5.7		0.413		
	9					0.722							
	10	30	7.0	2.93									
5 Stibisol	1	15	13.3	5.82	5.65	0.292	0.183			8.06		0.182	
	2	20	19.0	5.51	±0.90	0.209	±0.023			±1.82		±0.017	
	3	60	7.5	1.29		0.204		111	5.9		0.192		
	4	60	3.6	1.41		0.133		110	5.5		0.193		
	5	20	17.1	6.58		0.153		30	14.1		0.143		
	6	20	11.8	4.26		0.165		30	8.0		0.139		
6 Sodium anti mony ^{III} gluconate	1				6.29	0.331	0.331	26	6.6	6.53	0.400	0.314	
	2	20	8.4	3.21	±0.93	0.103	±0.054			±0.32		±0.035	
	3	90	5.6	1.06		0.396		105	6.7		0.327		
	4	20	14.6	4.69		0.325		30	6.9		0.309		
	5	20	8.4	4.46		0.278		20	5.7		0.197		
	6	20	5.2	3.64		0.377		20	7.1		0.259		
7 Solustibosan													
8 Sodium mannitol antimoniate													
9 Sodium anti mony ^V tartrate													
10 Stibophen (SbV)													
No activity in single doses													

No activity in single doses

TABLE 4—*Concluded*

TABLE 1—Continued

SUBSTANCE	EXPT.	HAWKING'S METHOD						DULBRING AND BURN'S METHOD					
		No. of mice used	b	σ_b	Weighted mean b	R.D. 50 (mg./20 gm.)	Weighted mean, R.D. 50	No. of mice used	b	Weighted mean b	S.E.D. (mg./20 gm.)	Weighted mean S.E.D.	
11. Neostam	1				8.85		4.13	27	4.7	8.02	4.95	3.71	
	2				± 1.36		± 0.30	50	5.8	± 1.88	3.64	± 0.24	
	3	58	7.6	1.81		3.80		78	7.3		3.47		
	4	30	7.6	2.81		4.88		40	5.6		4.00		
	5	20	10.2	4.09		4.94		30	9.4		3.76		
	6	20	18.9	6.53		3.61		20	14.6		2.99		
	7	20	18.6	6.52		3.63		20	17.0		3.20		
12. Neostibosan	No activity in single doses												
13. Ureastibamine	1				7.55		2.21	40	5.9	10.97	1.82	1.77	
	2	60	0.1	2.11	± 1.41	1.86	± 0.19	80	12.6	± 1.69	1.91	± 0.07	
	3							57	10.0		1.82		
	4	30	5.3	2.76		2.34		40	9.9		1.80		
	5	20	10.3	4.48		2.12		30	10.5		1.36		
	6	20	11.7	6.39		2.97		30	19.6		1.63		
	7	20	3.4	3.80		2.38		20	7.2		1.74		
14. Stibacetin	1	28	9.5	4.01	7.79	5.67	6.80	51	9.1	8.49	3.47	4.36	
	2	20	8.6	4.45	± 1.41	8.00	± 0.41	50	5.5	± 1.03	3.72	± 0.71	
	3	39	5.9	1.83		6.52							
	4	20	10.2	4.48		7.56		20	8.2		6.31		
	5	20	14.2	4.69		6.97		40	11.6		5.31		

Notes: Hawking's method—Mean value of b weighted for σ_b ($\omega = 1/\sigma_b^2$). Mean value of R.D.50 weighted for number of mice used in each determination.

Bulbring and Burn's method—Mean values of b and of S.E.D. weighted for number of mice used in each determination.

methods for the assay of trypanocidal activity shown in table 4 indicates that both methods give reproducible results, and that both have about the same variance. The differences between the results obtained by the two methods in the case of phenyl stibonic acid derivatives indicate that the methods measure different types of activity, and thus show that it is not possible to give a single figure to express the ratio of activities of a pair of substances so different in mode of action as tartar emetic and stibacetin.

When substances of similar chemical constitution are being compared, it makes little difference which method of assay is used.

The lack of trypanocidal activity shown by neostibosan and the quinquivalent analogues of tartar emetic has been further investigated and will form the subject of a future communication. Bulbring and Burn (11) found that a more pronounced action was obtainable with neostam if the drug was injected on 3 consecutive days, and all the substances listed here as inactive in single doses have been found to show activity if a large enough number of doses is given at short enough time intervals.

Although very little success has attended the use of antimony compounds in the treatment of human trypanosomiasis, the trypanocidal test has been extensively used as a means of selecting substances likely to be of use in other protozoal diseases. That this is an unsatisfactory procedure is well shown by the fact that

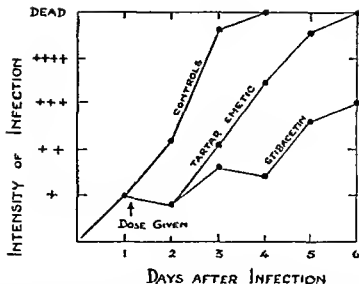


FIG 2 THE DEVELOPMENT OF A *T. equiperdum* INFECTION IN THE BLOOD OF GROUPS OF MICE DOSED WITH TARTAR EMETIC AND WITH STIBACATIN

The percentage of mice cleared of trypanosomes in the peripheral blood was the same in both groups, but the development of the infection was slower in the stibacatin treated mice

TABLE 5

The toxicity, irritant activity, trypanocidal activity and initial rate of excretion of antimony compounds in terms of metallic antimony

RESISTANCE	% Sb CONCENT	LD 50 (INTRAVEN) (MG Sb/20 GM)	SD 50 (SUBCUT) (MG Sb/20 GM)	MIN NECROSING CONC (% Sb)	% Sb EXCRETED IN 1 HOUR AFTER INTRAVENOUS DOSE
1 (b) Tartar emetic	39.0	0.363	0.0510	0.04	8.5
2 (b) Sodium antimony ^{III} tartrate	39.2	0.451	0.0561	0.08	13.0
3 Anthiomaline	16.0*	0.579	0.0528	0.16	0.0
4 Stibophen	13.5	4.21	0.0957	1.7	18.0
5 Stibsol	29.0	0.322	0.0531	0.03	10.0
6 Sodium antimony ^{III} gluconate	32.7	1.12	0.108	0.07	11.0
7 Sodium antimony ^V gluconate	26.3	8.68		3.3	20.0
8 Sodium mannitol antimoniate	23.4	23.9		>23.4	26.0
9 Sodium antimony ^V tartrate	27.0	1.39		3.4	24.0
10 Stibophen (Sb ^V)	13.4†	8.92		>6.7	20.0
11 Neostam	26.0*	7.67	1.07	0.13	15.0
12 Neostibosan	42.0*	3.96		0.21	14.0
13 Ureastibanine	40.0*	1.70	0.884	0.08	18.0
14 Stibacatin	32.2	1.82	2.19	0.32	14.0

* Manufacturers' published figures

† Assuming substance to be $C_{12}H_{10}O_{11}S_4Na_3Sb_7H_2O$

Most of the figures in the last column are taken from Goodwin & Page (1) for the intravenous dose of 3-4 mg Sb/kg. The rest of the figures were obtained from groups of mice also given doses containing 3-4 mg Sb/kg.

the best antimonials for the treatment of human leishmaniasis are inactive against *Trypanosoma equiperdum*. The trypanocidal test is useful for the routine control of samples of the same substance, and reveals interesting effects which

accompany changes in chemical constitution, but it should not be applied without reservation to the selection of drugs for the treatment of other diseases.

SUMMARY

1. A series of tervalent and quinquevalent organic antimonials has been tested for toxicity, irritancy and trypanocidal activity, and the results assessed by accurate methods.

2. Trypanocidal activity has been determined by two methods, one depending upon the removal of trypanosomes from the peripheral blood, and the other upon the survival times of infected mice. Differences have been observed between the results in the case of phenyl stibonic acid derivatives.

3. The properties investigated are shown to be independent of antimony content, but to have some correlation with one another and with the initial rate of excretion of the antimony.

The author wishes to thank Dr. J. O. Irwin for suggesting the use of expectations of life in the trypanocidal test; Dr. J. E. Page for the additional polarographic determinations of excreted antimony, and Mr. J. M. Judd for his invaluable assistance throughout the work.

REFERENCES

- (1) GOODWIN AND PAGE, *Biochem. J.*, **37**: 198, 1943.
- (2) GOODWIN AND PAGE, *Biochem. J.*, **37**: 482, 1943.
- (3) BROWN AND AUSTIN, *J. Amer. Chem. Soc.*, **63**: 2054, 1941.
- (4) WELLCOME FOUNDATION AND W. SOLOMON, *B.P.* 554317/42.
- (5) DURHAM, GADDUM AND MARCHAL, *Spec. Rep. Ser., Med. Res. Coun., Lond.* No. 128, 1929.
- (6) BLISS, *Quart. J. Pharm.*, **11**: 192, 1938.
- (7) PAGET, TREVAN AND ATTWOOD, *Int. J. Lep.* **2**: 149, 1934.
- (8) GADDUM, *Spec. Rep. Ser., Med. Res. Coun., Lond.*, No. 183, 1933.
- (9) HAWKING, *Quart. J. Pharm.*, **14**: 337, 1941.
- (10) HAWKING, *Quart. J. Pharm.*, **16**: 13, 1943.
- (11) BÜLBRING AND BURN, *Quart. J. Pharm.*, **11**: 67, 1938.
- (12) GRAY AND TREVAN, *Trans. R. Soc. trop. Med. Hyg.*, **25**: 147, 1931.
- (13) IRWIN, *J. R. Statist. Soc. Suppl.* **4**: 1, 1937.
- (14) BRUNNER, *Arch. Exp. Path. Pharmacol.*, **68**: 186, 1912.
- (15) BOCK, *Z. Hyg. Infectkr.*, **107**: 396, 1927.
- (16) FARGHER AND GRAY, *This Journal*, **18**: 341, 1921.
- (17) ROGERS, *Indian med. Gaz.*, **53**: 161, 1918.
- (18) BRAHMACHARI, *Indian J. med. Res.*, **10**: 492, 1922.
- (19) GRAY, TREVAN, BAINBRIDGE AND ATTWOOD, *Proc. roy. Soc. B.*, **108**: 54, 1931.
- (20) NAPIER, *In Kala-azar*, 2nd Edit., p. 145, Oxford University Press, Calcutta, 1927.
- (21) GUHA, DUTTA AND MUKERJI, *Nature*, **151**: 108, 1943.
- (22) CHUNG AND CHOW, *Chin. med. J.*, **61**: 73, 1942.
- (23) NAPIER, *Indian J. med. Res.*, **19**: 719, 1932.
- (24) BROWN AND AUSTIN, *J. Amer. vet. med. Ass.*, **48**: 566, 1939.
- (25) WEESE, *Chin. med. J.*, **52**: 421, 1937.
- (26) CHOPRA, *Indian J. med. Res.*, **15**: 41, 1927.
- (27) BRADLEY AND FREDRICK, *Industr. Med.* **10**: *Industr. Hyg. Sect.* **2**: 15, 1911.
- (28) UHLENHUTH, KUHN AND SCHMIDT, *Arch. Schiffs- u. Tropenhyg.*, **29**: 623, 1925.

A CONTRIBUTION TO THE PHARMACOLOGY OF THE ALIPHATIC AMINES

RAYMOND P AHLQUIST

From the Department of Pharmacology, Division of Pharmacy, South Dakota State College, Brookings

Received for publication April 1, 1944

The aliphatic amines were first studied extensively by Barger and Dale (1). It was found that sympathomimetic activity appeared in compounds with more than three carbon atoms. Maximum activity was present in the six carbon compound and thereafter decreased as the carbon chain was lengthened. These authors also noted that substitution in the amino group produced compounds of lesser activity.

In recent years some of these aliphatic amines have been found therapeutically useful. This study has been conducted on three that have proven of potential therapeutic value. These are I 2 methylamino iso-octene (Octin Bilhuber), II 2 methylamino heptane (EA-1 Bilhuber), III 2 amino heptane (Tuamine-Lilly).

Octin is useful as a smooth muscle spasmolytic especially in ureteral spasm (2) and Tuamine has been recommended as a nasal vasoconstrictor (3).

In a previous report (4) it was shown that Octin on intravenous injection in dogs produced a marked pressor response, the blood pressure rising rapidly and falling slowly. On repeated injections the pressor response becomes decreased with each subsequent dose (tachyphylaxis) and a preliminary pressure drop becomes increasingly apparent until with about the fifth dose no blood pressure rise occurs but only a fall. It has been postulated that this depressor effect is due to a direct depressant action on the myocardium.

Jackson (5) has described the action of EA-1 in dogs. The tachyphylactic action and increasing depressor response also occur with this drug.

In this paper the relationship between these amines as to their actions on blood pressure in dogs and their toxic effects on rats and frogs has been studied.

PRESSOR AND DEPRESSOR ACTION *Experimental Methods* Large, grossly normal dogs were anesthetized with ether. Blood pressure was recorded from the carotid artery by means of a mercury manometer. The drugs were injected into the femoral vein, a 1% aqueous solution of their water soluble salts being used. Atropine was administered to the animals to paralyze the vagi as tested by electrical stimulation.

The pressor response to single doses of each substance was determined in five dogs for each drug. Then the effect of rapidly repeated doses was determined, waiting between doses until the blood pressure had fallen to about the initial level. Alternate doses of the three drugs were tested in two animals.

RESULTS The depressor and pressor response to single 2 mgm per kgm doses of the various drugs is given in table 1. It will be seen that Tuamine produces the greatest pressor response and Octin the least. The depressor action

was most apparent with Octin and least with Tuamine. The duration of action was about the same for all, averaging about forty minutes.

The results of repeated doses of the drugs are given in table 2. These show that all of the compounds exhibit decreasing pressor action and increasing depressor activity. Octin shows the greatest depressor action.

TABLE 1
The depressor and pressor action of 2 mgm. doses of

OCTIN HCl		EA-1 HCl		TUAMINE SULFATE	
Dep *	Pr †	Dep	Pr	Dep	Pr.
14	130	0	160	0	150
12	152	28	120	10	120
0	100	6	110	0	140
14	110	0	150	6	160
10	122	0	120	8	120
Av. 10	123	7	132	5	138

* Depressor response in mm. of mercury.

† Pressor response in mm. of mercury.

TABLE 2
Depressor and pressor responses to repeated doses

DRUG	ANIMALS	REPEATED DOSES OF 2 MCGM PER KG									
		I		II		III		IV		V	
		Dep	Pr	Dep	Pr	Dep	Pr	Dep	Pr.	Dep	Pr
Octin HCl	5	14	119	45	64	57	32	67*	19	70*	0
Range.		±5	±39	±12	±34	±5	±18	±21	±17	±24	0
EA-1 HCl	4	12	125	19	56	31	29	35*	10	†	
Range..		±7	±40	±15	±22	±10	±20	±5	±10		
Tuamine sulfate	3	4	143	10	62	20	52	†			
Range.		±4	±20	±5	±7	±3	±5				

* Two dogs tested with these doses

† Animals died.

By administering 2 mgm. doses of the drugs alternately the results shown in table 3 were obtained. These show that the compounds are more or less interchangeable in their blood pressure effects. This is most clearly shown in Dog 41.

TOXICITY. I. RATS. Experimental Methods The LD-50 of these compounds was determined by intraperitoneal injection into male white rats weighing from 150 to 250 gms. To further test the interchangeability mixtures of certain percentages of the LD-50 were tested as to acute toxicity.

RESULTS The results of the determination of the LD 50 of these compounds are given in table 4. The symptoms produced by all three substances were identical. They include in the approximate order of appearance, mild tremors with excitement at about eight minutes after injection, severe tremors at about ten minutes followed by severe clonic convulsions with tremors between spasms. A general pilomotor reaction consisting of a bushing out of the hair is usually seen. After several convulsive spasms the animal appears depressed.

TABLE 3

Depressor and pressor response to alternate 2 mgm. doses

DOG	RESPONSE	A*	T†	O	A	T	O	A
40	Dep	28	10	40	54	20	40	60
	Pr	120	60	40	60	50	30	40
41	Dep		0		80		70	
	Pr		150		70		0	

* EA 1 hydrochloride

† Tuamine sulfate

‡ Octin hydrochloride

TABLE 4

Determination of LD-50 in rats

DOSE	OCTIN BITARTRATE	OCTIN HYDROCHLORIDE	EA 1 HYDROCHLORIDE	TUAMINE SULFATE
mgm /kgm	rats/d ed	rats/d ed	rats/d ed	rats/d ed
180	5/5	2/2	2/2	2/2
140	15/11	5/5		
130	10/5*	5/5		
120	10/2	2/2	2/2	
80	5/0	2/2		5/5
75		8/6	2/2	2/2
70		10/5†	5/4	5/4
60		5/0	10/5‡	10/5§
50			5/1	5/0

* LD 50 corresponding to 64 mgm/kgm of octin base

† LD 50 corresponding to 54 mgm /kgm of octin base

‡ LD 60 corresponding to 47 mgm /kgm EA 1 base

§ LD 50 corresponding to 42 mgm /kgm tuamine base

with the righting reflex absent. Death occurs about fifteen minutes after injection preceded by a short period of depression with gasping respiration. Death is probably due to respiratory failure. If the animal survives it gradually becomes more active and returns to normal in about one hour.

Octin Bitartrate is less toxic than Octin Hydrochloride when calculated as the dose of free base administered. This difference is probably due to a slower diffusion rate of the tartrate salt.

The results of administering mixed doses of these substances is given in table

5. Here again is clearly demonstrated the interchangeability of these compounds. This is a good example of almost absolute additive synergism.

II. FROGS. *Experimental Method.* Frogs were prepared according to Ilmer (6) with a glass chamber surrounding the heart. The drugs in various dilutions in Locke's Solution were placed in the chamber and the heart action recorded on a kymograph.

RESULTS. Little effect on amplitude was noted except when the rate fell to less than 30% of the initial rate. Then the amplitude rapidly decreased.

TABLE 5
Toxicity of mixed doses

OCTIN HCl	OCTIN BITARTRATE	EA-1 HCl	TUAMINE SULFATE	RESULT
	% of LD-50			rats/died
50		50		10/5
75		25		10/6
25		75		10/4
	50	50		10/5
		50	50	10/6

TABLE 6
Action on rate of frog heart

DRUG	CONC	PER CENT OF INITIAL RATE AT GIVEN INTERVALS IN MINUTES AFTER APPLICATION*								
		2	4	6	8	10	15	20	25	30
Locke's solution	%	98	97	97	100	98	91	91	91	90
Octin HCl	05	98	110	110	100	98	98	90	90	88
Octin HCl	1	100	80	73	65	65	50	45	40	34
Octin HCl	2	74	66	61	55	50	16	0		
EA-1 HCl	05	98	110	100	95	90	98	88	85	88
EA-1 HCl	1	100	88	76	67	62	47	41	35	32
EA-1 HCl	2	76	42	37	32	32	32	21	15	0
Tuamine SO ₄	1	100	80	80	70	70	65	65	50	45
Tuamine SO ₄	2	80	65	65	60	50	43	33	15	10

* Average results for 3 to 5 frogs with each drug and concentration.

In table 6 are shown the effects of different concentrations of these drugs on the heart rate. These results indicate that with the lowest concentration a slight increase in rate appeared. With the higher concentration only slowing was produced. Tuamine being the least active as to depressant properties and Octin the most active. This correlates well with the depressor activity shown by these drugs.

CONCLUSIONS AND SUMMARY

From the results obtained it appears that the only difference in vasomotor activity and toxicity of these three amines is in degree. The activity seems

to vary with molecular weight rather than molecular configuration. The pressor action in dogs and the acute toxicity in rats varies inversely as the molecular weight, while the depressor effect in dogs and myocardial depression in frogs varies directly as the molecular weight.

Of interest also is the apparent interchangeability of these compounds. This would suggest that they all have the same mode and point of action.

The author wishes to thank the Bilhuber-Knoll Corporation for supplying the Octin and EA-1 for this work and also wishes to thank Marguerite Hartheb for technical assistance.

REFERENCES

- (1) BAROER, G , AND DALE, H H , Jour Physiol , **41** 19, 1910
- (2) LUND, H G , AND ZINGALE, F G , Jour Urol , **50** 65, 1943
- (3) PROETZ, A W , Ann Otol , Rhin & Laryng , **51** 112, 1942
- (4) AHLQUIST, R P , J Am Pharm Assoc , **32** 151, 1943
- (5) JACKSON, D E , Jour Lab and Clin Med , **29** 150, 1944
- (6) HINER, L D , Am Jour Pharm Ed , **3** 78, 1939

STUDIES ON SHOCK INDUCED BY HEMORRHAGE VIII. THE INACTIVATION OF THE APOENZYME OF AMINO ACID OXIDASE AND LACTIC DEHYDROGENASE IN ANOXIA¹

MARGARET E. GREIG

*From the Department of Pharmacology, Vanderbilt University School of Medicine,
Nashville, Tennessee*

Received for publication April 10, 1944

While investigating the destruction of coenzymes in shock induced by hemorrhage and in anoxia we compared the rate of amino and lactic acid oxidation by various excised tissues after preliminary incubation in oxygen or in nitrogen. It was found that preliminary incubation of the tissue in nitrogen produced a marked decrease in its ability to oxidize these substrates. Analyses for coenzyme, the coenzyme required for the oxidation of lactate, and alloxazine adenine dinucleotide, the coenzyme required for amino acid oxidation, showed only slight differences between the anoxic and normal tissues. Moreover, addition of these coenzymes to anoxic tissue, while producing an increase in the rate of oxidation, did not increase it to the normal value.

Evidence is here presented to show that the apoenzyme, or protein part of the enzyme, is inactivated under anaerobic conditions and in damaged tissue by a heat labile, enzyme like substance.

METHODS. *Tissues.* Tissues of albino rats and of the common variety of pigeon were used.

Slices were made by the method of Deutseh (1) and homogenate was prepared by snipping the tissue with scissors and grinding it with Krebs' phosphate buffer in a glass homogenizer.² Tissue extracts contained 1 gram of homogenized tissue in a total volume of 10 cc.

Enzymes. *d-Amino acid oxidase.* The apoenzyme and coenzyme, alloxazine adenine dinucleotide, were both prepared by the method of Warburg and Christian (2).

Lactic dehydrogenase. The apoenzyme was prepared by the method of Green and Brosteaux (3) and coenzyme by that of Williamson and Green (4).

Determination of coenzymes. Tissues in which coenzymes were to be determined were heated at 100° for 5 minutes. If they had not been homogenized for the experiment preceding the determination they were homogenized after treatment at 100°, then centrifuged and the supernatant fluid diluted appropriately for use in the analysis.

Alloxazine adenine dinucleotide (A.A.D.) was determined by using the purified apoenzyme of Warburg and Christian, as already described (2).

Coenzyme was determined by the method of Axelrod and Elvehjem (5). The preparation of the apoenzyme used in this analysis has already been described (6).

Measurements of oxygen uptake were carried out in Warburg manometers at 37°.

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Vanderbilt University.

This paper was released for publication April 4, 1944.

² Obtained from Scientific Glass Apparatus Company, Bloomfield, New Jersey.

RESULTS In table 1 it will be seen that anoxia produces a marked decrease in the ability of pigeon liver homogenate and rat kidney slices to oxidize dl alanine, l glutamate and lactate

To obtain some indication as to whether the decrease in oxidative ability was caused by destruction of coenzymes, alloxazine adenine dinucleotide, cozymase and cocarboxylase were added to the normal and anoxic tissues (table 2) The addition of these coenzymes although improving the rate of oxidation of the

TABLE 1

Comparison of rates of oxidation of various substrates by normal tissues and tissue previously subjected to anoxia

(Control values i.e., values obtained without added substrate subtracted)

TISSUE	WEIGHT IN EACH VESSEL	CONDITION OF TISSUE	SUBSTRATE	DURA- TION OF EXPER- IMENT	MM ³ O ₂ ABSORBED IN EXCESS OF CONTROL VALUE (WITHOUT SUBSTRATE)		
					Normal t issue	Anox c t issue	% de- crease in anox c t issue
Pigeon liver	333	Homogenized	dl alanine	60	275	130	53
Pigeon liver	333	Homogenized	dl alanine	60	147	104	29
Pigeon liver	350	Homogenized	dl lactate	50	374	127	66
Pigeon liver	406	Homogenized	l glutamate	60	168	35	77
Pigeon liver	354	Homogenized	l glutamate	60	128	30	77
Pigeon liver	506	Homogenized	l glutamate	50	250	62	75
Rat kidney	187	Slices	dl alanine	70	190	130	32
Rat kidney	187	Slices	l glutamate	60	255	80	60
Pigeon liver	314	Homogenized	dl lactate	60	255	35	86
Pigeon liver	310	Homogenized	l glutamate	60	930	200	78
Rat kidney	150	Slices	dl alanine	60	450	62	86

Krebs phosphate buffer was used in these experiments. The final concentration of substrate was 0.08 M. Total volume 2 cc.

The anoxic tissue was incubated in an atmosphere of nitrogen the normal homogenate in air and normal slices in oxygen for one hour before substrate was added. The nitrogen was then replaced by air or O₂ depending upon whether homogenate or slices were used and measurements of O₂ uptake were taken.

The duration of the experiment given in the table applies to the time during which O₂ uptake was measured.

anoxic tissue, did not restore its activity to that of the normal. These experiments do not conclusively disprove that coenzyme destruction is responsible for the decreased oxidative capacity in the anoxic tissue since it is possible that the coenzymes did not diffuse readily into the cell. However, analyses for cozymase and A.A.D. of normal and anoxic tissues showed only slight differences although large decreases in cozymase were produced on homogenization (table 3).

The accumulation of intermediate metabolites may furnish an alternative explanation for the decrease in amino acid and lactate oxidation by tissue previously exposed to anoxia.

To avoid this possibility the effect of tissue extracts on the isolated enzyme systems, d-amino acid oxidase and lactic dehydrogenase was determined (tables 4 and 5). The extracts were diluted to such extent that the concentration of any metabolites would be greatly reduced compared with that in the whole tissue.

TABLE 2

Effect of addition of coenzymes on the rate of oxidation of substrates by normal and anoxic tissues

(Control values subtracted)

TISSUE	WEIGHT IN EACH VESSEL	CONDITION OF TISSUE	SUBSTRATE	DURA- TION OF EXPER- IMENT	COENZYMES ADDED	MM ³ O ₂ ABSORBED IN EXCESS OF CONTROL VALUES	
						Normal	Anoxic
Pigeon liver	mg 384	Homogenized	l-glutamate	min 60	A A D *†	128 520	30 80
Pigeon liver	346	Homogenized	l-glutamate	60	coccarboxylase cozyme, A A D	930 730	200 310
Pigeon liver	346	Homogenized	dl-lactate	60	coccarboxylase cozyme, A A D	210 200	85 95

* A A D = alloxazine adenine dinucleotide

† 1 mg each added

TABLE 3

Coenzyme content of normal and anoxic tissues

TISSUE	COENZYME DETERMINED	COENZYME IN γ /GRAM MOIST TISSUE		
		At start	After incubation in	
			Air	N ₂
Pigeon liver	Cozyme	570	52	108
Pigeon liver	Cozyme		176	147
Pigeon liver	Cozyme		70	72
Pigeon liver	Cozyme	1050*	127	116
Pigeon liver	A A D		51	51
Pigeon liver	A A D		47	47

* Heated at 100° before homogenizing.

Inhibition of both enzyme systems was produced by extracts of various tissues. The inhibitor was thermolabile and non-dialyzable (tables 4 and 5). Krebs (7) found a similar inhibitor in kidney extracts.

Destruction by tissue extracts of alloxazine adenine dinucleotide has been

TABLE 4

Effect of tissue extracts on the activity of the reconstructed d-amino acid oxidase system

All vessels contained 0.2 cc 4.5% dl alanine, 0.1-0.2 cc alloxazine adenine dinucleotide (10-20 γ), phosphate buffer to make the final volume 2 cc

	MM ³ O ₂ ABSORBED	DURATION OF EXPERIMENT min
0.5 cc enzyme	490	60
+ 0.2 cc liver extract	365	
+ 0.2 cc boiled liver extract	480	
+ 0.2 cc brain extract	575	
+ 0.2 cc boiled brain extract	540	
0.2 cc brain extract	0	
0.2 cc liver extract	0	
0.5 cc enzyme	460	60
+ 0.2 cc liver extract	345	
+ 0.2 cc supernatant liver extract*	335	
+ 0.2 cc brain extract	460	
+ 0.2 cc supernatant brain extract*	475	
0.5 cc enzyme	225	60
+ 0.2 cc liver extract	155	
+ 0.2 cc brain extract	255	
+ 0.2 cc kidney extract	200	
+ 0.2 cc HCN (M/25)	275	
+ 0.2 cc liver extract + 0.2 cc HCN	177	
+ 0.2 cc kidney extract + 0.2 cc HCN	265	
0.5 cc enzyme	375	120
+ 0.2 cc liver extract	308	
+ 0.2 cc kidney extract	400	
+ 0.2 cc HCN	430	
+ 0.2 cc liver extract + 0.2 cc HCN	342	
+ 0.2 cc kidney extract + 0.2 cc HCN	555	
0.5 cc enzyme	373	60
+ 0.2 cc liver extract	256	
+ 0.2 cc supernatant liver extract*	268	
+ 0.2 cc muscle extract	346	
+ 0.2 cc supernatant muscle extract*	341	
+ 0.2 cc supernatant heart extract*	357	
0.5 cc enzyme	386	60
+ 0.2 cc dialyzed liver extract	216	
+ 0.2 cc dialyzed heart extract	262	
+ 0.2 cc heart extract	266	

* Supernatant fluid from centrifuged tissue extract

reported by Ochoa and Rossiter (8) and of coenzyme by Mann and Quastel (9) and by von Euler and coworkers (10). In our reconstructed d-amino acid oxidase and lactic dehydrogenase systems we added large excesses of coenzymes

However, to prove that destruction of A.A.D. by liver extract was not sufficient to account for the decreased rate of oxidation of dl-alanine by d-amino acid oxidase, the experiments recorded in table 6 were performed. It will be seen that when the coenzyme was incubated with liver extract at 37° for one hour after which the inhibitor was destroyed by heat, and enzyme and substrate then added, the rate of oxidation was the same as when A.A.D. not treated with liver

TABLE 5

Effect of tissue extracts on the activity of lactic dehydrogenase

(All vessels contained 0.5 cc. M cyanide, 0.1 cc. 0.5% methylene blue, 0.1 cc. coenzyme (300 γ), 0.2 cc. 2M lactate, and M/5 phosphate buffer pH 7.4 to make the total volume 2 cc.)

	MM ³ O ₂ ABSORBED	DURATION OF EXPERIMENT
		min.
0.5 cc. enzyme ...	220	85
+ 0.2 cc. liver extract.	112	
+ 0.4 cc. liver extract . .	67	
+ 0.2 cc. boiled liver extract	205	
+ 0.2 cc. brain extract	174	
+ 0.4 cc. brain extract	140	
+ 0.2 cc. boiled brain extract	210	
0.5 cc. enzyme	235	90
+ 0.1 cc. liver extract	165	
+ 0.2 cc. liver extract	90	
+ 0.2 cc. dialized liver extract	140	
+ 0.4 cc. dialized liver	88	
0.5 cc. enzyme	160	85
+ 0.1 cc. muscle extract	135	
+ 0.2 cc. muscle extract	130	
+ 0.1 cc. heart extract	142	
+ 0.2 cc. heart extract	92	
0.5 cc. enzyme	143	60
+ 0.1 cc. brain extract	100	
+ 0.2 cc. kidney extract	145	
+ 0.2 cc. heart extract	83	
+ 0.1 cc. pancreas extract	130	
+ 0.2 cc. pancreas extract	53	

extract was used. It, therefore, appears that the inhibitory effect exerted by tissue extracts is exerted on the apoenzyme.

Attempts were made to purify the apoenzyme of d-amino acid oxidase, after it had been attacked by the liver inhibitor, by precipitation by means of $\frac{1}{2}$ saturation with ammonium sulphate and acidification to pH 2.8 according to the method of Warburg and Christian, but it was found that the inhibitor was also precipitated and remained active after this treatment.

The inhibition produced by liver extract is not abolished by cyanide. Cyanide, however, increases the activity of d amino acid oxidase, perhaps by combining with α keto acids formed during the reaction (tables 4 and 5)

The effect of brain extract on the two enzyme systems is different. With d amino acid oxidase it produces an acceleration and with lactic dehydrogenase an inhibition. Extracts of other tissues investigated act similarly with both enzyme systems.

Liver extract, when added to pigeon liver homogenate, also inhibits the oxidation of lactate and of glutamate (table 7), so the effect is not peculiar to isolated enzyme systems.

TABLE 6

Effect of preliminary incubation of A A D with liver extract on rate of oxidation of dl alanine by d amino acid oxidase

	MM ³ O ₂ ABSORBED	
	Experiment I	Experiment II
0.2 cc liver extract + 0.2 cc A A D in buffer at 37°C for 1 hour, heated at 100° cooled and added enzyme + dl alanine	472	450
0.2 cc A A D in buffer at 37°C for 1 hour heated at 100°, cooled and added enzyme + dl alanine	467	455
0.2 cc A A D in buffer at 37°C for 1 hour, heated at 100°, cooled and added enzyme + dl alanine + liver extract	365	353

TABLE 7

Effect of tissue extracts on oxidation of added substrates by liver tissue

SUBSTRATE	LIVER EXTRACT	MM ³ O ₂ ABSORBED IN 60 MIN
l glutamate		208
l glutamate	0.2 cc	82
Lactate		250
Lactate	0.2 cc	197

DISCUSSION It has been found that in excised tissue made anoxic by preliminary incubation in an atmosphere of nitrogen, a decrease in the ability to oxidize amino and lactic acids occurs. The inhibition is partially, but not completely, overcome by the addition of the coenzymes, cozymase, alloxazine adenine dinucleotide and co-carboxylase.

In order to investigate further the factor which causes this inhibition in oxidative processes the assumption is made that it is the same as that which occurs in damaged tissue and which has been found to inhibit the activity of the isolated enzyme systems d amino acid oxidase and lactic dehydrogenase. This assumption would seem reasonable in view of the similar enzymatic destruction of co-carboxylase which occurs in both anoxic and damaged tissues (11).

This factor inhibits lactic dehydrogenase and amino acid oxidase by attacking the apoenzyme or protein part of the enzyme system. It is heat labile and non-dialyzable. It is possibly an enzyme, although it does not belong to the group of enzymes which Keilin and Hartree (12) found would inhibit d-amino acid oxidase since its effect is not abolished by cyanide. It seems to be liberated under anoxic conditions and by tissue damage or to be produced by these conditions from an inactive precursor.

We have already shown that in shock induced by hemorrhage the coenzymes, coenzyme A, coenzyme B and alloxazine adenine dinucleotide, may be destroyed (13, 6). It seems possible that in this condition, and in others where anoxia and cellular damage occur, this enzyme like substance which destroys the apoenzymes would come into play, and thus further reduce the ability of the tissue to metabolize normally. There is some evidence for destruction of the apoenzyme in shock in the *in vitro* work of Russell et al. (14) on shocked tissue. They found a decreased ability of kidney slices of shocked rats to metabolize glucose which was benefited but not restored to normal by the addition of *Kochsaff*. On the other hand, Klein (15) has found that thyroid feeding produces an increase in activity of the d-amino acid oxidase of rat liver which is probably due to an increase in the protein component of the oxidase.

The factor found in anoxic and damaged tissues probably is not specific for d-amino acid oxidase and lactic dehydrogenase but may attack other apoenzymes under similar conditions.

SUMMARY

1. Tissues which have been made anoxic show a decreased ability to metabolize lactic and amino acids.

2. This decreased oxidative ability is not due entirely to coenzyme destruction nor to an anaerobic accumulation of intermediate metabolites which inhibit oxidation.

3. A heat labile, non-dialyzable, enzyme like substance has been found in tissue extracts which inhibits lactic and amino acid oxidation. It is assumed that this is the same factor which inhibits oxidation in anoxic tissue.

4. This enzyme like substance appears to attack the apoenzyme or protein part of the oxidative enzyme systems.

REFERENCES

- (1) DEUTSCH, J. *Physiol.*, **87**: 56P (1936).
- (2) WARBURG AND CHRISTIAN, *Biochem. Z.*, **298**: 150 (1938).
- (3) GREEN AND BROSTEAUX, *Biochem. J.*, **30**: 1489 (1936).
- (4) WILLIAMSON AND GREEN, *J. Biol. Chem.*, **135**: 345 (1940).
- (5) AXELROD AND ELVEHJEN, *J. Biol. Chem.*, **131**: 77 (1939).
- (6) GREIG, M. E., In press, *THIS JOURNAL*.
- (7) KREBS, *Biochem. J.*, **29**: 1620 (1935).
- (8) OCHOA AND ROSSITER, *Biochem. J.*, **33**: 2008 (1939).
- (9) MANN AND QUASTEL, *Biochem. J.*, **35**: 502 (1941).

- (10) VON EULER MYRDÄCK AND BRUNIUS *Z Physiol Chem* **177** 237, 183 60 (1929)
VON EULFR AND GÜNTHER *Z Physiol Chem* **243** 1 (1936)
VON EULER HEIWINKEL AND SCHLENK *Z Physiol Chem* **247** IV (1937)
- (11) GRIG AND GOVIER *THIS JOURNAL* **79** 246 (1943)
- (12) KEILIN AND HARTREE *Proc Roy Soc* **119B** 114 (1935-36)
- (13) GOVIER AND GREIG, *THIS JOURNAL* **79** 240 (1943)
- (14) RUSSELL LONG AND WILHELM, *J Exper Med*, **79** 23 (1944)
- (15) KLEIN *J Biol Chem* **131** 139 (1939)

THE BONE MARROW PROCEDURE FOR THE ASSAY OF LIVER EXTRACTS FOR ANTI-PERNICIOUS ANEMIA ACTIVITY

C. M. YOUNG AND H. D. BETT

From the Connaught Laboratories, University of Toronto, Toronto, Canada

Received for publication April 13, 1944

A laboratory method for the determination of potency of liver extracts intended for the treatment of pernicious anemia would be of great value. Numerous unsuccessful attempts have been made to devise such a procedure. Overbeek and associates (1, 2) have described a method involving the use of explants of bone marrow from normal guinea pigs. When these were cultured in a solid medium of saline and heparinized plasma, cells, chiefly leucocytes, migrated from the explants to form a reasonably clear-cut zone of migration. Little, if any, cell division occurred. Addition of an active liver extract in a series of dilutions was said to stimulate the migration, not as a general effect, but to give a maximum value at one definite dilution for each extract, of the order of 1:100. The effect was stated to be duplicated for the same extract at a dilution approximating 1:10,000. An example of the effect is shown in Figure 1 by the single solid line. These maximal migrations will be referred to subsequently as "peak values."

A modification of this procedure was utilized by Pace and Fisher (3), who reported that the method could be used quantitatively. Following the suggestion of Overbeek et al., Pace and Fisher carried out their work in the higher dilution range, so that commercial and other extracts containing preservatives might be tested. Sodium oxalate was used as an anti-coagulant in place of heparin, and clotting of the plasma was brought about by the addition of Ringer's solution containing calcium. As a result of experiments using these modifications they reported that the activity of different extracts could be represented as a linear function of the dilution at which peak migration occurred, and presented a graph relating this dilution and activity in U.S.P. units.

An attempt to use the procedure of Pace and Fisher, both as described and with a number of modifications, has been made in this laboratory. At the beginning of the work the details of the procedure were personally discussed with Dr. Fisher, to whom thanks are expressed for extensive assistance.

METHOD. Solutions: A. 0.1 M. sodium oxalate; B. Ringer's solution: 0.152 M. sodium chloride, 0.003 M. potassium chloride, 0.002 M. calcium chloride, 0.0009 M. sodium monohydrogen phosphate, and 0.0001 M. potassium dihydrogen phosphate. C. As in B, with calcium chloride omitted.

The following was carried out with sterile technique. Blood was drawn from the exposed heart of an anaesthetized guinea pig weighing about 250 grams with a syringe containing 0.1 cc. of sodium oxalate for each cc. of blood. After centrifugation the plasma was drawn off and diluted with twice its volume of solution C.

The femurs were removed, scraped clean, and the bone marrow taken out and cut into pieces about 1 mm. in diameter. Five or six of the pieces were put into each dish containing 0.75 cc. of solution B for the controls or of one of a series of dilutions of liver extract in solu-

tion B. One quarter of 1 cc. of diluted plasma was added, well mixed, and the pieces arranged in the dish. The calcium in the Ringer's solution (B) was sufficient to cause the formation of a firm clot in five to ten minutes. The dishes were covered with tightly-fitting lids. When set, the cultures were incubated at 37 degrees for 15 hours.

At the end of that time the dishes were placed in a projector and the outlines of the original explant and of the outer limit of the migration area were traced on squared paper. These areas were estimated, designated O_1 and O_2 respectively, and the relative growth (M) found from the relation $(O_2 - O_1)/O_1$. The M values for each dish were averaged, and the mean divided by the mean of M for all the controls and multiplied by 100 to give the figure "P", or percental migration coefficient. P was plotted against dilution to demonstrate the presence or absence of a peak.

About forty tests were carried out using the method exactly as outlined. The results given in table 1 include only those obtained with extracts which had been clinically tested, all but No. 104 were clinically active. Extracts referred to by a number were prepared in this laboratory.

TABLE 1
Results of tests by method of Pace and Fisher

EXTRACT	NUMBER OF TESTS	PEAK OBTAINED	NO PEAK	DOUBTFUL
93	1	1		
100	7	2	3	2
104	2		1	1
105	3	1	2	
106	4	1	3	
108	1	1		
Brand A, commercial, 10 unit	4	1	1	2
Brand B, commercial, 15 unit	1	1		

In these first experiments the dilutions used were generally within the range where a peak might be expected, according to the clinical activity of the extracts and the relationship given by Pace and Fisher. For example, an extract containing 10 U.S.P. units per cc. was tested in several experiments over a range of dilution from 1:45,000 to 1:150,000. From their curve, these limits would correspond to 6 and 19 units respectively. Accordingly, a peak occurring anywhere except at the extremes of the range might be considered as confirmation not only of the occurrence of a response, but also of the quantitative relationship which they describe.

As some cultures migrated to only a small extent and the cell concentration was often not dense enough for satisfactory projection, the growth in Ringer's solution was compared with that in glucosol, a modified Tyrode's solution (4). This closely resembles Ringer's solution except for the presence of magnesium and glucose. The same sample of bone marrow appeared to give slightly denser growth in glucosol, which was accordingly substituted for the Ringer's solution. Subsequent experiments showed no difference that might be attributed to the

change in medium. Again only the results obtained with clinically tested extracts are presented.

Altogether, from thirty-three experiments on extract 100, an extract of high clinical activity, eight peaks were obtained at dilutions equivalent to 3, 11, 14, 20, 21, 23, 27 and 33 U.S.P. units according to the curve of Pace and Fisher. In thirteen cases no peak was obtained. Extract 104, an extract shown to be clinically inactive, failed to produce any peak in thirteen out of twenty experiments; but peak migration as definite as any obtained with active extracts did occur in four cases, at dilutions equivalent to 2, 13, 14 and 18 U.S.P. units, again as estimated from the curve of Pace and Fisher.

The erratic occurrence of a peak was further shown in a set of four experiments using extract 100, with which several peaks had been obtained at dilutions approximating 1:200,000. Twelve dilutions were used between 1:100,000 and 1:230,000. Bone marrow from two or three pigs was cut up, well mixed, and thirty pieces selected for each dilution, six in each dish. In each experiment ten dishes were used as controls. The procedure and solutions used in the four experiments were the same, except for the use of different bone marrow.

TABLE 2
Results of tests by modified method

EXTRACT	NUMBER OF TESTS	PEAK OBTAINED	NO PEAK	DOUBTFUL
100 (active)	24	6	10	8
109 (active)	14	4	3	7
104 (inactive)	18	4	12	2

The mean relative migration (M) for the controls varied between experiments from 18 to 24. The P value was found for each dilution, and the four P values were averaged to give the value plotted in figure 1 as a double line, each point on which represents from 85 to 120 pieces of bone marrow. This figure approximates a straight line, with limits of 100 to 125, a variation which is not significant. However, in one of the four experiments a well-defined peak occurred, as shown by one single line, on which each point represents the mean of over 20 pieces of bone marrow. The other single line represents an experiment in which no peak was found.

Growth of the bone marrow fragments took place even when no liver extract was present. Variation in the M value of 285 pieces of bone marrow grown in seventy-one dishes in glucosol and plasma alone was very marked, the mean for the individual pieces being 18 ± 6.2 , and for groups of pieces in separate dishes 18 ± 4.5 . The mean M value for a single dish in at least four cases was sufficiently above the mean of all the dishes to be considered a "peak".

Several modifications were tried in an attempt to reduce the variability of the controls. The use of rat bone marrow, which might be expected to be more uniform than that from guinea pigs, and of heterologous plasma (rabbit), as well as the calculation of values representing difference in area ($O_2 - O_1$) or pro-

portional to difference in diameter ($\sqrt{O_2} - \sqrt{O_1}$) instead of relative growth $(O_2 - O_1)/O_1$ were all tried. None effected any improvement.

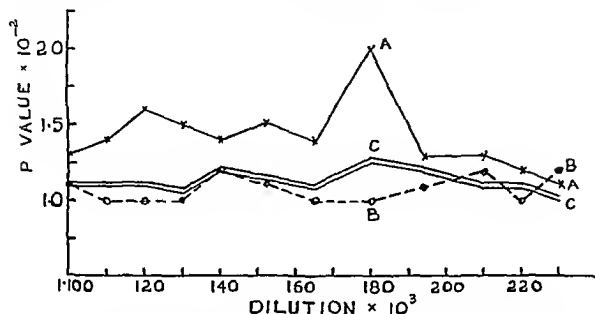


FIG 1 GRAPH SHOWING THE INCONSISTENT OCCURRENCE OF PEAKS WITH A SERIES OF DILUTIONS OF EXTRACT No 100

- A Single experiment showing peak (Experiment No 337)
 B Single experiment showing no peak (Experiment No 339)
 C Mean of four experiments

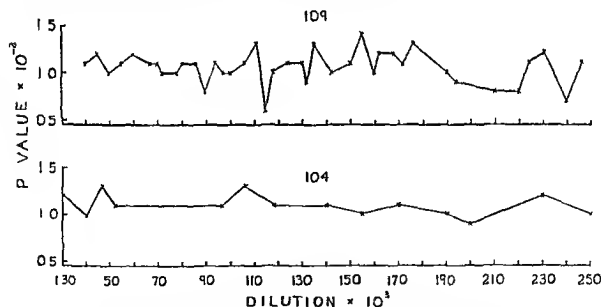


FIG 2 COMPARISON OF P VALUES OBTAINED WITH AN ACTIVE EXTRACT (109) AND AN INACTIVE EXTRACT (104) OVER A SERIES OF DILUTIONS

Except in one series of experiments on extracts 100 and 104 there was little regular deviation over a series of dilutions ranging from 1:100 to 1:300,000 from the straight line representing the controls, and it is extremely probable from the conditions of those experiments that an apparent parabolic curve obtained by plotting P against dilution for extract 100 in this particular series was an artefact.

No such feature occurred with any other active extract tested, or with extract 100 in subsequent experiments.

Comparison of extracts 104 and 100, and 104 and 105, with and without the content of organic solids adjusted to the same level, showed no difference in either case. The addition of folie acid to extract 104 equal to the amount present in extract 105 caused no stimulation of growth.

DISCUSSION. The occurrence of peak migration of cells from normal guinea pig bone marrow in a medium consisting of plasma, saline, and active liver extract; as reported by Overbeek et al., has also been shown in our work. For this reason it seems that the substitution of oxalate or citrate for heparin as an anti-coagulant, an important difference in the methods, does not prevent the development of peak migrations. Similarly, it would appear that the use of a higher dilution of liver extract, which they suggested, and at which they reported a peak as occurring, has not been responsible for the discrepancy between our findings and theirs.

The results presented here show that similar peaks may be obtained with inactive extracts and with no liver extract added to the medium. The occurrence of peak migration does not appear to be specific for active liver extracts, nor is the dilution at which it occurs quantitatively related to the clinical activity of extracts. The marked variation in the growth of different pieces of bone marrow under the same conditions seems in itself to be sufficient to account for the occurrence of peaks, though if only a few tests were performed the presence of peaks might be considered as indicative of activity of the liver extract, and the erratic nature of their appearance might be overlooked. In addition, the response of normal guinea pig or rat bone marrow cultured in guinea pig, rat, or rabbit plasma does not appear to vary to any marked extent with the addition of liver extracts, active or inactive, or of folie acid in the quantity present in liver extract. This is true both over a series of dilutions and for any one dilution within the range of 1:100 to 1:300,000, as long as the number of fragments used is sufficient to overcome the effect of the great variation in the marrow itself.

SUMMARY

Using the method of Pace and Fisher, adapted from that of Overbeek et al., we have been unable to confirm their reports that a quantitative or qualitative relationship exists between the occurrence of a "peak" migration of cells from normal guinea pig bone marrow grown in a medium of saline, plasma, and liver extract and the activity of the extracts. Peak migration does not take place consistently at any dilution from 1:100 to 1:300,000 of extracts of known anti-pernicious anemia activity, but occurs irregularly whether or not any liver extract is present. There is little, if any, added stimulation by purified extracts above that taking place in plasma and salt solutions; the graph of growth-concentration appears to be approximately a straight line with zero slope in all cases. No difference sufficiently marked to be used as the basis of an assay method seems to exist between the response to active and to inactive extracts, or to extracts of differing clinical activity. No definite relationship has been demon-

strated between response and anti pernicious anemia activity, content of organic solids, or content of folic acid

This work was under the supervision of Dr E W McHenry. Many of the assays were carried out by D P Joel and Miss Grace Tripp. To these and other members of the staff of the Connaught Laboratories who contributed to these experiments we wish to extend our thanks.

REFERENCES

- (1) GAILLARD, P J, OVERBEEK, G A, AND YAM, T H, *Arch Internat de Pharmacodyn et de Therap*, **64** 33, 1940
- (2) OVERBEEK, G A, GAILLARD, P J AND DE JONGH, S E, *Schweiz Med Wehnschr* **68** 711, 1938.
- (3) PACE, N, AND FISHER R S, *THIS JOURNAL* **74** 256, 1942
- (4) PARKER, R C, *Methods of Tissue Culture*, Hoeber, 1938, New York
- (5) EBELING, A H, *J Exper Med*, **34** 231, 1921

A TOXICOLOGICAL AND PHARMACOLOGICAL INVESTIGATION OF SODIUM SEC-BUTYL ETHYL BARBITURIC ACID (BUTISOL SODIUM)¹

CHARLES M. GRUBER, FRED W. ELLIS AND GOLDIE FREEDMAN

From the Department of Pharmacology, Jefferson Medical College, Philadelphia

Received for publication April 17, 1944

The first work on sec-butyl ethyl barbituric acid of which published results are available was done by Shonle and Moment (1) in 1923 who noted the subcutaneous toxic dose for rabbits to be less than 0.2 gm. per kg. Tabern and Shelberg (2) gave to this barbiturate an efficiency rating better than the ratings of diethyl or of phenyl ethyl but slightly lower than that of ethyl (1-methyl butyl) barbituric acid. These efficiency ratings were based upon the comparative solubilities of these drugs in fats and in water. Using 100 gm. rats, Shonle, Kelteh and Swanson (3) studied the relative toxicities of a number of barbiturates subcutaneously injected. They found the "M.L.D." for diethyl to be 400; phenyl ethyl, 240; isoamyl ethyl, 180; n-butyl ethyl, 200; and sec-butyl ethyl, 140 mg. per kg. While these findings give an accurate comparison of the toxicities of these drugs in their particular investigation, since they define "the M.L.D." as "that dose which caused the death of all or a majority of the animals on that dose," it is not possible to compare these results with the M.L.D. given by other investigators. The first careful investigation of the relative toxicities of a number of barbiturates was made by Fitch and Tatum (4). They determined the "minimal lethal dose" in rabbits and rats for these barbiturates when they were administered either orally or intraperitoneally. They found the LD₅₀ for sec-butyl ethyl barbiturate for rabbits to be 75 mg. and 140 mg. per kg. respectively when given intraperitoneally and orally. The intraperitoneal LD₅₀ for rats they found to be 70 mg. per kg.

Inasmuch as the sodium salt of sec-butyl ethyl barbiturate (butisol sodium) is being used in clinical medicine, an investigation on experimental animals of its toxicological and pharmacological actions is due. Experiments were therefore performed in which the effects of sodium sec-butyl ethyl barbiturate (butisol sodium) were compared with the effects of sodium diethyl barbiturate (barbital sodium), sodium phenyl ethyl barbiturate (phenobarbital sodium), and sodium ethyl (1-methyl butyl) barbiturate (pentobarbital sodium). The toxicology studies were carried out on 50 dogs, 300 rabbits, 600 albino rats and 590 white mice. Five per cent solutions were used in the experiments on dogs and rabbits and 1 and 2 per cent solutions in rats and mice. In the toxicity studies on dogs the injections were slowly made intravenously. In the rabbits the drugs were administered intravenously at a constant rate of 62.5 mg. (1.25 cc.) per minute and in albino rats the intravenous injection rate was 8 mg. (0.8 cc.) per minute.

¹ This research work was made possible through a grant from the McNeil Laboratories.

In rabbits and albino rats the oral and intraperitoneal toxicities were also determined. In white mice only intraperitoneal injections were made.

The animals were given a drug but once in determining the LD_{50} except in two instances in which small doses had caused no deaths in a group of animals. These animals were therefore used again three weeks later as a group for testing the effect of a larger dose of the same drug.

TABLE 1

In this table the weights of dogs and rabbits are expressed in kg and those for rats and mice in gm

BARBITURATE	ANIMAL	WEIGHTS		METHOD OF ADMINISTRATION	LD ₅₀ mg per kg
		Extremes	Average		
Sodium sec butyl ethyl (Butisol sodium)	Dog	7 - 14.5	10.5	Intravenous	90
		17 - 30	23	Intravenous	91
	Rabbit	1.5 - 2.3	1.9	Intraperitoneal	95
		1.2 - 2.0	1.7	Oral	194
	Albino rat	110 - 150	123.0	Intraperitoneal	76
			165.0	Intraperitoneal	70
		102 - 155	124.0	Oral	78
		152 - 187	175.0	Intravenous	70
	White mouse	17 - 23	20.5	Intraperitoneal	247
Sodium phenyl ethyl (Phenobarbital sodium)	Rabbit	1.5 - 2.4	1.9	Intravenous	185
	Albino rat	110 - 146	124.0	Intraperitoneal	190
	White mouse	18 - 23	21.0	Intraperitoneal	340
Sodium ethyl (1 methyl butyl) (Pentobarbital sodium)	Albino rat	140 - 230	185.0	Intraperitoneal	48
		112 - 139	124.0	Intraperitoneal	75
	White mouse	16 - 20	18.0	Intraperitoneal	140
Sodium diethyl (Barbital sodium)	White mouse	17 - 28	21.0	Intraperitoneal	763
Sodium ethyl n butyl (Neonal sodium)	Albino rat	112 - 151	126.0	Intraperitoneal	197

The rabbits, rats and mice were divided into batches of 12 to 25 animals each. The results immediately above and below the LD_{50} included two or more such batches for each drug studied. A summary of the results is presented in table 1. It will be noted that the intravenous, intraperitoneal and oral toxic doses for sodium sec butyl ethyl barbiturate are approximately the same for the albino rat. In the rabbit the intravenous and intraperitoneal toxic doses are approximately the same whereas the oral LD_{50} is about twice as high. This difference in the results on the rat and rabbit may be due to the fact that the rabbit's stomach is not empty even though the animal has fasted 48 hours. The

presence of gastric contents in the stomach would dilute the drug and consequently delay its absorption, thus decreasing its toxic effect.

In the albino rat the intraperitoneal dose which we found necessary to kill 50 per cent of the animals is slightly higher than that found by Fitch and Tatum (4). This small difference may be due to the fact that they used the acid whereas we used the sodium salt. In rabbits, however, the difference between our intraperitoneal and oral doses and the doses they cite cannot be attributed to the same thing. It is possible that sizes of the animals used in the two series of experiments was the chief factor. Our animals were small, weighing between 1.2 and 2.3 kg. and the groups averaged but 1.9 and 1.7 kg. Unfortunately the weights of the animals used by Fitch and Tatum are not given. Small animals usually tolerate proportionately larger doses of a barbiturate than do larger ones.

Assays were done on samples of sodium sec-butyl ethyl barbiturate, using the method outlined in the U.S.P. under pentobarbital sodium. The residues from each of these assays contained 92.6 per cent of the sodium salt used. The melting points of the residues as well as that of a sample of sec-butyl ethyl barbituric acid were between 165° and 165.5°C. This is somewhat higher than that found by Shonle and Moment (1) (155° to 157°C.) but approximately the same as that found by Shonle, Kelteh and Swanson (2) (164° to 165°C.).

Our experiments on rats with pentobarbital sodium support the findings of Fitch and Tatum (4). Carmichael (5), on the other hand, found the median lethal intraperitoneal dose of nembutal to be 110 to 120 mg. per kg. for young rats and 85 to 94 mg. per kg. for old rats. Our LD₅₀ for animals averaging 124 gm. in weight was 75 mg. per kg. and for older animals averaging 185 gm. it was 48 mg. per kg. We are unable to account for the discrepancy between our results and those of Carmichael.

Pentobarbital sodium (nembutal) and three other labeled U.S.P. brands of pentobarbital sodium were studied. With three of the brands* 66 mg. per kg. caused a mortality of approximately 60 per cent in 80 animals injected, whereas with a fourth preparation, although labeled U.S.P., the same dose injected into 31 rats not only caused no deaths but even failed to produce hypnosis in a single instance. One week later these same animals were again injected with the same preparation with the same results. Three weeks later these animals were given 66 mg. per kg. of pentobarbital sodium (Gane's) and 66 per cent of them died. Two U.S.P. assays were done on each of the four pentobarbital sodium preparations. In the three efficacious preparations the residue contained 88.6 to 90 per cent of the pentobarbital sodium used. These residues had melting points between 127° and 129°C. which is within the range allowed by the U.S.P. The residue of the two assays of the fourth preparation yielded 84.6 and 86 per cent of the original pentobarbital sodium taken. The melting point of this residue was 172.5°C. The lack of toxicity of this fourth preparation was therefore

* These three brands of pentobarbital sodium were supplied to us by Abbott Laboratories, Eli Lilly & Company and McNeil Laboratories, whom we wish to thank. The preparation sent to us by the McNeil Laboratories was made by Gane's Chemical Works.

undoubtedly due to its not being of USP quality, although labeled as such. From this it would seem wise to assay all barbiturates before toxicity studies are undertaken.

In this study the criterion of the duration of action was much the same as that used by Fitch and Tatum (4). The animals were checked at the time they went to sleep and again as soon as they could raise their heads, sit upright, maintain that position and hop or walk about when disturbed. The rats and mice were counted awake as soon as they could right themselves and crawl around. As the animals are still definitely depressed, the actual periods of the barbiturate depression are, of course, much longer than the times secured by

TABLE 2

Average increase in the duration of the depressant action of sodium sec butyl ethyl barbituric acid with increasing dosage

ANIMAL	DOSE mg per kg	LD ₅₀ per cent	AVERAGE DURATION OF ACTION	
			hr	min
Rabbit				
48	36	40	2	3
10	45	50	3	11
7	75	83	5	43
Dog				
2*	18	20	1	58
9†	22	25	2	17
18	30	30	5	48
18	36	40	7	28
Albino rat				
14	30	40	2	27
62	40	53	3	33
10	50	70	4	3

* Four animals failed to respond by hypnosis

† Three of the twelve animals given this dose did not respond to the drug by hypnosis

these methods. All of the animals were watched continuously from the time of the injection until they were checked off as awake. Our experimental animals responded to sodium sec butyl ethyl barbiturate in much the same way as they did to other barbituric acid derivatives. The duration of action varied with the animal employed. Large animals appeared to be more depressed than smaller ones of the same species when the same dose per kg was administered. Healthy animals were less depressed than sick ones and they were depressed for a shorter time. Only the results of healthy animals are included in this report. Within limits the larger the dose the longer the depression.

It will be seen from table 2 that dogs are more susceptible to the drug than are either rabbits or rats. In 18 dogs 40 per cent of the LD₅₀ caused an average depression of over seven hours' duration whereas the same percentage of the

LD₅₀ caused an average depression of only a little over two hours in 48 rabbits and 14 rats studied.

That sodium sec-butyl ethyl barbiturate is more rapid in action than either barbital sodium or phenobarbital sodium but less rapid than pentobarbital

TABLE 3

Duration of action and the time required for the barbiturate to produce hypnosis. Intraperitoneal injections were made in the mouse and rat. Sixty per cent of the LD₅₀ was used and all injections were 1 per cent solutions except those of diethyl in the mouse which was a 2 per cent solution

The injections were made intravenously at a constant speed in rabbits. In these animals a 5 per cent solution was used and the amount injected was 50 per cent of the LD₅₀. The sodium salt was used in each case.

ANIMAL	MG. PFR KG.	BARBITURATE	INDUCTION TIME		DURATION OF ACTION	
			Extremes	Average		
			min.		hr.	min.
Mouse	84	Ethyl (1-methyl butyl)	2-4	2.7	1	47
	148	Sec-butyl ethyl	3-6	4.0	8	15
	457	Di-ethyl *	13-27	21.0	9	23
	204	Phenyl ethyl †	11-45	21.0	17	52
Albino rat	150	n-hexyl ethyl	3-5	4.0	1	44
	29	Ethyl (1-methyl butyl)	2-3	2.5	2	25
	118	Ethyl n-butyl	4-13	6.0	3	37
	45	Sec-butyl ethyl	5-12	8.0	3	55
	114	Phenyl ethyl ‡	16-30	23.5	3	23
	60	Propyl-methyl-carbinyl allyl	2-6	2.6	5	10
Rabbit	23	Ethyl (1-methyl butyl)	§		1	2
	45	Sec-butyl ethyl	§		2	10
	93	Phenyl ethyl	5-27	13.0	6	11
	22.5	Propyl-methyl-carbinyl allyl	§		1	2
	40	Iso-amyl ethyl	§		2	38

* One of the fifteen animals failed to show hypnosis and one required 51 minutes for the development of depression which lasted but one hour and 15 minutes.

† One animal failed to show depression and 4 of the 15 animals died. The depression varied from 4 to 36 hours.

‡ Three of the 15 animals failed to show hypnosis.

§ Animals completely depressed at end of injection.

sodium can be seen in table 3. In duration of action it holds a similar relationship to these official derivatives of barbituric acid.

The most favorable dose of sodium sec-butyl ethyl barbiturate causing hypnosis varied in the different species used. In dogs and rabbits 40 per cent and in rats 60 per cent of the LD₅₀ proved to be the most favorable doses. We determined the minimal hypnotic doses (M.H.D.) for rabbits and dogs. We used the same criterion as that employed by Werner, Pratt and Tatum (6), as follows: "The M.H.D. is that amount of drug in milligrams per kilogram which

caused 50 per cent or more of the animals receiving that dose to lie on their sides with head down." These changes were noted in 83 per cent of the rabbits tested when 23 mg per kg were injected intravenously. The calculated therapeutic coefficient is therefore $\frac{M L D}{M H D} = \frac{91}{23} = 4$. This figure is slightly lower than that found by Werner, Pratt and Titum (6) for pentobarbital sodium, 4.5, but higher than that of phenobarbital sodium which was found to be $\frac{190}{57} = 3.3$.

Twelve dogs were given 22 mg per kg of sodium sec butyl ethyl barbiturate intravenously. Nine of the animals showed hypnosis lasting from 17 minutes to 4 hours and 40 minutes. From these results it appears that in the dog the calculated therapeutic coefficient is 3.9. Only those animals which received large toxic doses of sodium sec butyl ethyl barbiturate appeared to have a "hangover" the next day.

In addition to the experiments described above, others were performed on 10 dogs anesthetized with ether. In these a solution of sodium sec butyl ethyl barbiturate was injected intravenously. Simultaneous records were made of the effect of the injection on the respiration, the blood pressure and the volume of one or more organs. The blood pressure was recorded by means of a mercury manometer using heparin in the system as the anti coagulant. The respirations were recorded by means of a large recording tambour connected to a pneumograph placed about the thorax of the animal. The volume changes of the spleen and kidney or leg were recorded by means of oncometers connected to modified Brodie bellows.

RESPIRATION. Like other barbiturate acid derivatives sodium sec butyl ethyl barbiturate depresses respiration. The degree of depression is dependent upon the amount of the drug administered. In our non anesthetized animals used in studying the toxicity of the preparation 30 to 60 per cent of the LD_{50} would decrease both the rate and depth of respiration. In some instances the rate was reduced to one fifth that of the control and in some animals a typical Cheyne Stokes respiration was observed.

A similar depression of respiration was noted following the intravenous injection of the drug in dogs anesthetized with ether. In all of these experiments the ether was discontinued before the injection of the drug. In the experiment illustrated by figure 1, 30 mg per kg (30 per cent of LD_{50}) were injected intravenously at 1. Immediately after the injection respiration stopped for 30 seconds. On resumption of breathing the respiratory rate was 30 per minute instead of 48. Large doses rapidly administered caused complete and permanent cessation of respiration although the heart continued to beat for some minutes longer.

CIRCULATION. The effect of the drug on the arterial blood pressure is dependent upon the amount of the drug administered and upon the speed of the injection. If the drug is injected rapidly in large doses a marked fall in blood pressure results. In some of our experiments 30 mg per kg rapidly injected caused drops in blood pressure of over 100 mm Hg. In figure 1 30 mg per

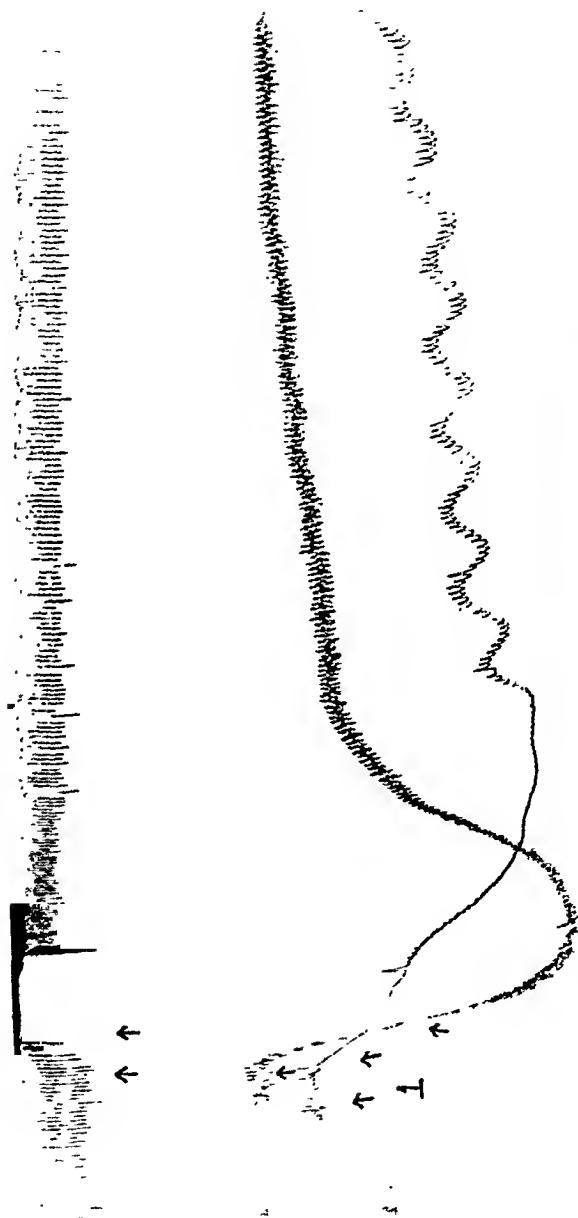


Fig. 1. MALE DOG, 10.5 Kg.

Ether anesthesia. Top record is of respiration in which the down stroke is inspiration and below it the carotid blood pressure taken with a mercury manometer. Bottom record, the time in intervals of 30 seconds and zero blood pressure and above it a record of changes in the volume of the spleen. At I, between the arrows, 30 mg. per kg. of sodium sec-butylethyl barbiturate were rapidly injected intravenously.

kg of sodium sec butyl ethyl barbiturate were injected rapidly at 1. The arterial blood pressure dropped from 145 to 38 mm Hg, after which it gradually returned to the control level. The fall in blood pressure is, as far as we have been able to determine, due to a dilatation of the blood vessels of the skin, extremities, spleen and other organs. Our plethysmographic experiments show that if the fall in blood pressure is not too precipitous the spleen, the limb, the ear and the kidney increase in size. Figure 2 is a record selected from a series of experiments as being typical of the responses of the spleen to slow injections of sodium sec butyl ethyl barbiturate. At 1, 30 mg per kg of the drug were injected intravenously, as a result of which the volume of the spleen increased and the blood pressure temporarily decreased (135 to 84 mm Hg). As said before, in other experiments similar responses were observed in the limb, the ear and the kidney.

Inasmuch as these same changes were seen in the limb with its nerve supply cut, we infer that the vasodilatation is due to a direct action of the drug on the smooth muscle.

If the drop in blood pressure is sudden and extensive, decreases in the volumes of the limb, kidney and spleen are seen (see figure 1). This decrease in size we believe is a passive phenomenon.

Except in the animal used in figure 2 spontaneous premature ventricular contractions were not encountered in any of the experiments done on 10 dogs. This irregularity in this case had been present during the time the animal was under ether anesthesia before the administration of sodium sec butyl ethyl barbiturate and its frequency was not influenced by repeated injections of the drug. Like other barbiturates this one probably does not change the irritability of the heart muscle to any extent in the doses used.

In two animals sodium thiopentobarbital was injected intravenously in small doses and in both runs of premature ventricular contractions lasting for several minutes were produced which could be made to disappear suddenly by the injection of either glyceryl trinitrate or epinephrine hydrochloride (7).

INTESTINE. The effects of varying concentrations of sodium sec butyl ethyl barbiturate on excised longitudinal segments of rabbit and cat intestine were also studied. These effects were compared with those obtained by the use of other barbiturates on the same segments. All of the animals were killed by cerebral concussion and exsanguination. Some of the tissues were used immediately after being removed while others were kept in a refrigerator in cold Locke's solution until needed.

One end of the muscle was fastened to an L-shaped glass rod and the other end to a muscle lever by means of ligatures. The muscles thus suspended, together with the L-shaped glass rod, were placed in a pyrex glass cylinder containing freshly made Tyrode's solution with a pH of 7.8. Two muscle segments, one of duodenum and one of ileum, were suspended in the same bath. The temperature of the bath was maintained at $38.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ by surrounding the cylinder containing the tissues with a large volume of water (a glass aquarium) kept in motion by an electrically driven circulator and warmed by an electric

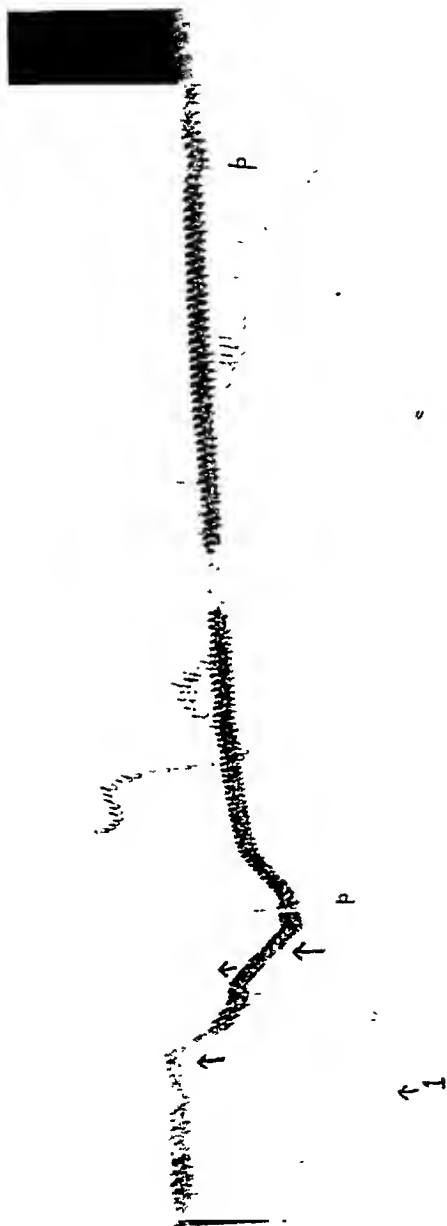


FIG. 2. MALE DOG, 22.5 Kg.

The animal received an injection of sodium sec-butyl ethyl barbiturate one hour before this record was made. Top record the arterial blood pressure taken from the left carotid artery with a mercury manometer. Bottom record the time in intervals of thirty seconds and zero blood pressure. Middle record that of the change in volume of the spleen. At 1, between the arrows, a second intravenous injection of 30 mg. per kg. of sodium sec-butyl ethyl barbiturate was made. Two premature contractions of the ventricle may be seen in the blood pressure record at *p*. These contractions occurred before the administration of the drug while the animal was under ether anesthesia and are hence not due to the sodium sec-butyl ethyl barbiturate. The barbiturate appeared to have no influence on their frequency.

heating element controlled by a thermo regulator and relay. The bath was oxygenated by a stream either of oxygen or of air bubbling through it. A kymograph and chionographic marking key which recorded intervals of thirty seconds were employed.

The drugs were weighed and dissolved in Tyrode's solution just before each experiment. Adjustments were made to maintain a constant pH. A given amount of this solution was added to the bath to make the necessary dilutions which ranged from 1:100,000 to 1:2,000. After exposure of the intestinal segments to sodium sec butyl ethyl barbiturate for two minutes the Tyrode's solution containing the drug was drained off, the segments washed and immersed in fresh Tyrode's solution. Upon complete recovery of the tissue from the effects of the drug the process was repeated with phenobarbital sodium, pento barbital sodium and sodium n butyl ethyl barbiturate. One hundred and ninety two such experiments were performed on 18 pairs of segments of rabbit intestine and 51 experiments were done on 10 pairs of segments of cat intestine. Within the limits of our experiments phenobarbital sodium appeared to be approximately half as depressant and pentobarbital sodium twice as depressant as sodium sec butyl ethyl barbiturate. This action can be seen in figure 3. At 2 phenobarbital sodium was added to the bath making a dilution of 1:5,000, at 4 sodium sec butyl ethyl barbiturate 1:12,500, and at 6 pentobarbital sodium 1:25,000. The depressant effect of the sodium sec butyl ethyl barbiturate is slightly greater than that caused by phenobarbital sodium though less than that caused by the more dilute pentobarbital sodium solution. Sodium n butyl ethyl barbiturate (Neonal) at 5 appears slightly more depressant than sodium sec butyl ethyl barbiturate at 6 with the same concentration. These relative differences appeared in all our experiments.

The effect of sodium sec butyl ethyl barbiturate was also studied on the intact intestine. Four healthy, trained non anesthetized dogs weighing between 12 and 18 kg. with Thury Vella loops were employed. Of these animals one had a duodenal, one a jejunal and the other two had ileal fistulae. The changes in the general tonus and in the rhythmic contractions were recorded by means of a rubber balloon placed within the lumen of the gut (8). The balloon was connected to a modified Brodie bellows. The pressure within the balloon was 15 cm. of water. After a normal control record had been secured the drug was injected intravenously in doses of 30 mg. per kg. or 30 per cent of I.D.₅₀. Twelve such experiments were performed. Marked relaxation of the general tonus of the gut was noted following each injection as illustrated by figure 4. In this experiment a dog weighing 18 kg. with a Thury Vella loop of the jejunum was given intravenously at 1 the barbiturate dissolved in distilled water. Decreased general tonus of the gut resulted almost immediately, lasting for approximately one hour. In not a single experiment was the decrease in the general tonus of the gut followed by an increase above that of the control level.

UTERUS. Similar experiments were also performed on segments of excised cat, rabbit and guinea pig uterus. Two longitudinal segments, one from each horn of the uterus, were suspended as the strips of intestine had been and the

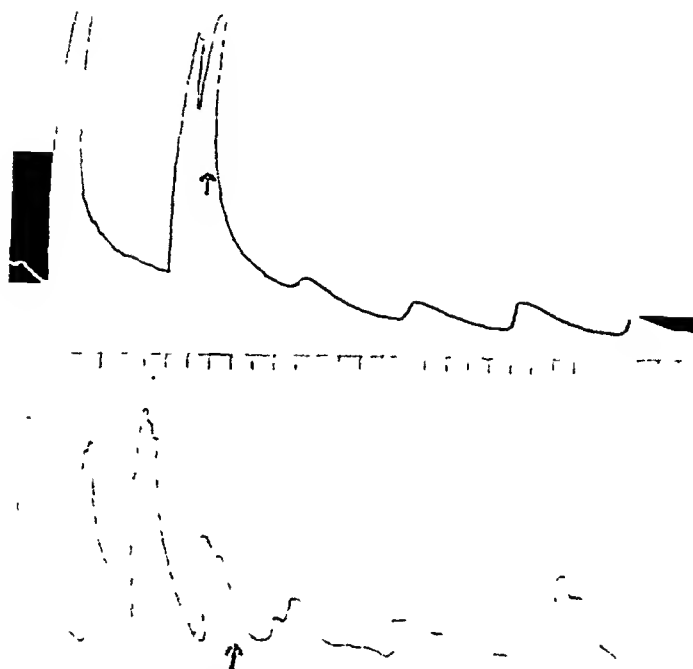


FIG. 5 EXCISED LONGITUDINAL SEGMENTS OF RABBIT UTERUS

Time in 30 second intervals. At the arrows sodium sec-butyl ethyl barbiturate was added to the bath to make a 1:5,000 solution.

TABLE 4

Duration of action of sodium sec-butyl ethyl barbiturate before and after removal of the kidneys

The control readings were taken three weeks before nephrectomy. Fifteen hours after excision of both kidneys the same dose of the drug was administered as in the control.

WEIGHT	LD ₅₀	CONTROL TIME	TIME AFTER NEPHRECTOMY
kg	per cent	min	min
9.5	25	251	202
7.0	25	117	103
9.0	25	184	200
10.5	30	270	300
11.0	30	270	297
9.0	40	390	383
9.5	40	375	405

methods were otherwise the same except that Locke's solution was used instead of Tyrode's solution. The dilutions of the drug varied from 1:2,000 to 1:10,000. These concentrations uniformly caused decreases in both the height of the

rhythmical contractions and the general tonus. In figure 5, nt the arrows, sodium sec-butyl ethyl barbiturate was added to the bath to make a 1:5,000 solution. Depression of both segments was produced immediately.

CHRONIC TOXICITY. For many months we gave rabbits and rats respectively 40 and 60 per cent of LD_{50} every other day for a period of two weeks alternated with a rest period of two to three weeks. Upon examination of these animals after death no gross changes in the liver and other organs were observed. Young animals were used and these continued to gain in weight during the entire period.

EXCRETION. Only when large toxic doses of sodium sec-butyl ethyl barbiturate were administered to dogs and rabbits could the drug be detected in the urine by the Koppanyi test (9).

To determine whether or not the drug is eliminated entirely by the kidneys experiments were performed in which the duration of action of the drug was determined before and after nephrectomy (10). Seven dogs were used in this study as seen in table 4. To establish controls each animal was given a certain per cent of the LD_{50} per kg. three weeks before the kidneys were removed and the period of the depression timed. Fifteen hours after nephrectomy the animals were given a similar dose and again the duration of depression noted. It will be seen in table 4 that removal of the kidneys produced no significant increase in the duration of action of the drug. From these results it must be assumed that the drug is detoxified or destroyed somewhere in the body, probably in the liver.

SUMMARY

1. The intravenous LD_{50} for sodium sec-butyl ethyl barbiturate for dogs, rabbits and albino rats was found to be 90, 91 and 70 mg. per kg. respectively. The intraperitoneal doses for rabbits, albino rats and white mice are 95, 70 to 76, and 247 mg. per kg. respectively. The oral dose for rabbits is 194 and for rats 78 mg. per kg.

2. The intravenous LD_{50} for rabbits for phenobarbital sodium is 185 mg. per kg. and the intraperitoneal doses for rats and mice are 190 and 340 mg. per kg. respectively.

3. The intraperitoneal LD_{50} for pentobarbital sodium in rats weighing 124 gm. is 75 and in those weighing 185 gm. it is 48 mg. per kg. (supporting Fitch and Tatum (4) but not Carmichael (5)). In mice it is 140 mg. per kg.

4. The intraperitoneal LD_{50} for barbital sodium in white mice is 763 mg. per kg.

5. The intraperitoneal LD_{50} for sodium ethyl n-butyl barbiturate in albino rats is 197 mg. per kg.

6. The duration of action of sodium sec-butyl ethyl barbiturate is dependent upon the total amount of the drug injected and upon the size, health and species of animal used. Of the animals used in this study it is longest in the dog.

7 For the three species of animals employed the barbiturates which we studied can be arranged in the following order according to their durations of action: Mouse, (1) pentobarbital sodium; (2) sodium sec-butyl ethyl barbiturate;

(3) barbital sodium; (4) phenobarbital sodium. *Albino rat*, (1) n-hexyl ethyl; (2) ethyl (1 methyl butyl); (3) phenyl ethyl; ethyl n-butyl; sec-butyl ethyl; (4) propyl-methyl-carbinyl allyl. *Rabbit*, (1) ethyl (1-methyl butyl), propyl-methyl-carbinyl allyl; (2) sec-butyl ethyl; (3) iso-amyl ethyl; (4) phenyl ethyl.

8. A large dose of sodium sec-butyl ethyl barbiturate rapidly injected intravenously will produce either marked slowing or even permanent cessation of respiration. The heart continues to beat some minutes longer.

9. Administration of sodium sec-butyl ethyl barbiturate will cause a fall in arterial blood pressure. The extent of the fall is dependent upon the amount given and the speed of injection or absorption.

10. If the fall in blood pressure is not extensive it is accompanied by an increase in the volume of the spleen, limb, kidney and ear. This vasodilator action is due to a direct effect of the drug on the vessel wall.

11. Sodium sec-butyl ethyl barbiturate, as far as could be determined, produces no change in cardiac rhythm such as is seen in the dog following sodium thiopentobarbital.

12. The excised as well as the intact intestine is depressed by sodium sec-butyl ethyl barbiturate. In the excised intestine its depressant effect is somewhat greater than that of phenobarbital sodium but less than that produced by pentobarbital sodium.

13. Excised uterine segments of rabbits, cats and guinea pigs are depressed by this barbiturate.

14. No gross changes in the liver and other organs were noted after a prolonged series of administrations of the drug with rest intervals between.

15. No significant difference was found in the duration of action of the drug in normal and nephrectomized dogs. It is assumed therefore that the drug is destroyed somewhere in the body and partly excreted in the urine only when given in excessive doses.

REFERENCES

1. SHONLE, H. A., AND MOMENT, A., J. Am. Chem. Soc., **45**: 213, 1923.
2. TABERN, D. L., AND SHELBERG, E. F., J. Am. Chem. Soc., **55**: 328, 1933.
3. SHONLE, H. A., KELTCH, A. K., AND SWANSON, E. E., J. Am. Chem. Soc., **52**: 2410, 1930.
4. FITCH, R. H., AND TATUM, A. L., THIS JOURNAL, **44**: 325, 1932.
5. CARMICHAEL, E. B., THIS JOURNAL, **52**: 284, 1938.
6. WERNER, H. W., PRATT, T. W., AND TATUM, A. L., THIS JOURNAL **60**: 189, 1937.
7. GRUBER, C. M., GRUBER, C. M., JR., AND COLOSI, N., THIS JOURNAL, **60**: 143, 1937; Am. J. Obstet. and Gyn., **33**: 729, 1937; Gruber, C. M., Haury, V. G., and Gruber, C. M., JR., THIS JOURNAL, **63**: 193, 1938; Kohn and Lederer, J. Lab. Clin. Med., **23**: 717, 1938.
8. GRUBER, C. M., AND BRUNDAGE, J. T., THIS JOURNAL, **53**: 120, 1935.
9. KOPPANYI, DILLE, MURPHY AND KROP, J. Am. Pharm. Assoc., **23**: 1074, 1934; Proc. Soc. Exp. Biol. and Med., **30**: 542, 1933; Arch. internat. de Pharmacod. et d. Therap., **46**: 76, 1933.
10. HIRSCHFELDER, A. D., AND HAURY, V. G., Proc. Soc. Exp. Biol. and Med., **30**: 1059, 1933.

CLINICAL ACTIONS OF ETHYLNORSUPRARENIN

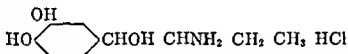
M L TAINTER, M D, W M CAMERON, M D, L J WHITSELL, M D AND M M HARTMAN, M D¹

From the Departments of Pharmacology and Therapeutics, and of Medicine, Stanford University School of Medicine San Francisco

Received for publication May 1, 1944

There is no doubt that epinephrine is the most generally useful of all the sympathomimetic amines. Improvements in therapy during the 40 years use of this group of compounds have come through development of products in which some one action, or group of actions, is stronger than that of the parent substance, thus permitting a more specialized application. A good example is amphetamine (benzedrine) which causes such marked central nervous system stimulation as to permit the drug to be used with little consideration of its sympathomimetic actions.

A compound which appears to us to have possibilities of useful therapeutic application is 1-(3,4 dihydroxyphenyl) 2 amino 1 butanol, which we have described in various papers under the somewhat misleading name of ethylnor suprarenin. In the form of the racemic hydrochloride salt, used in this study, it is a colorless, odorless crystalline powder with a bitter taste. It is readily soluble in water and alcohol and insoluble in ether and benzene. It melts with decomposition at 198-200°C.



In animals this drug does not cause the sharp pressor effects of epinephrine but rather decreases the diastolic pressure. As a result the mean pressure is decreased, the pulse pressure increased, and the pulse accelerated. Aside from this, the compound has many of the actions of epinephrine, and therefore, might be used under circumstances where pressor effects would not be beneficial, and in doses not limited by the danger of pressor crises. This report presents the results of certain clinical studies which indicate a definite field of usefulness for this drug.

RÉSUMÉ OF EXPERIMENTAL RESULTS This compound was originally synthesized in the laboratories of the I G Farbenindustrie Hoechst, Frankfurt am Main, and supplied to us by Dr O Schaumann, who, however, apparently did not publish any data on it. In 1933, one of us (T) reported that the compound, in doses of 0.3 mgm per kilo intravenously, lowered the mean blood pressure in anesthetized cats and accelerated the pulse (1). A year later it was reported from this department (2) that the compound dilated rather effectively the bronchi.

¹ Supported, in part, by Therapeutic Research Grant 437 of the Council on Pharmacy and Chemistry, American Medical Association, and by grants from the Rockefeller Fund Research Fund of Stanford University School of Medicine.

of perfused guinea pig lungs, when previously constricted by histamine, barium chloride, or pilocarpine. The dose required for this was much greater than with epinephrine, but the degree of relaxation was practically identical. In intact dogs, in which bronchoconstriction was recorded by Jackson's technic and produced by injections of arceoline, 1 mgm. per kilogram of ethylnorsuprarenin relaxed the bronchi in one-half the animals with equivocal results in the remainder (3). However, the low blood pressure in these dogs was increased by the compound instead of the depressor response observed at normal levels. In a later paper (4), where the same technic was used with histamine to cause the bronchospasm, the drug proved to be a good bronchodilator, although here it failed to overcome the lowering of blood pressure.

In the intact cat the effects on the circulation were quite complex (5). After intravenous injection the drug caused a sharp fall of mean arterial pressure with acceleration of the heart and relaxation of peripheral vessels. The depressor effect was secondarily enhanced by pooling of blood in the splanchnic region. The cardiac output was increased, but, since the mean peripheral resistance was decreased, this probably was not accompanied by a corresponding increase in cardiac work. In a later paper (6), it was shown that on repeated injections at short intervals, pressor effects could be produced, apparently by sensitization of sympathetic vasoconstrictors. However, under ordinary conditions, ethylnorsuprarenin was demonstrated to stimulate predominantly both the cholinergic and adrenergic vasodilators, and therefore to cause the fall of systemic blood pressure primarily by relaxing the blood vessels. It was pointed out that this would result in diminishing the load of peripheral resistance, against which the heart ordinarily pumps the blood.

In phenol poisoning in cats, no improvement of the condition of the circulation was observed after the administration of ethylnorsuprarenin, at least as far as the blood pressure level was concerned, but no measurements of volume flow of blood were made (7). In the shock state caused by destruction of the central nervous system, the compound produced little effect, possibly because, with the peripheral vessels already well dilated from the loss of vasomotor impulses, the main effects of the drug could not be elicited (8). The compound apparently had no analeptic power in rats against the narcotic actions of avertin, chloral or pentobarbital (9), nor in producing hyperexcitable states in unanesthetized rats (11). Rats tolerated 80 mgm. per kilo subcutaneously without fatalities, but were killed at the 160 mgm. dose level. With epinephrine, a similar proportion were killed by 4 mgm. per kilo, indicating a toxicity about one-fortieth that of epinephrine. In perfused cat's legs, when several hundred times the dose of ethylnorsuprarenin was injected as is required of epinephrine to produce constriction, a constrictor effect could be demonstrated (10). But in such preparations the vasomotor tonus is low, so that the primary actions of the former are not elicited.

In summary, these data showed that ethylnorsuprarenin increased cardiac activity and dilated peripheral vessels, thereby promoting the flow of blood, that it effectively relaxed bronchi, and that it lacked demonstrable excitant

actions on the central nervous system. These results made it desirable to investigate its actions in patients to establish whether it had useful effects on the circulation and bronchi, and whether these would be accompanied by less undesired sympathomimetic reactions than with equivalent doses of epinephrine. This paper describes mainly the results of clinical tests in patients.

INTRAVENOUS FATAL DOSE² The previously reported work did not include an accurate determination of the fatal doses whereby a comparison of the acute toxicity of the new compound with epinephrine could be obtained. Accordingly, white mice were injected rapidly in the tail vein with 0.5 cc of normal salt

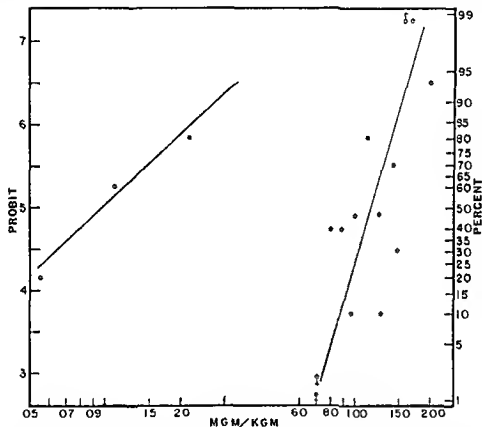


FIG 1 PERCENTAGE MORTALITY IN WHITE MICE FROM EPINEPHRINE (LEFT) AND ETHYLNORSUPRARENIN (RIGHT) INJECTED INTRAVENOUSLY

(LD₅₀ epinephrine 0.98 ± 0.184 and ethylnorsuprarenin 117 ± 1.04 mgm per kg)

solution containing the required dose of one of the amines, administered in proportion to the body weight. The epinephrine was the levo bitartrate, and the ethylnorsuprarenin the racemic hydrochloride, but the doses were calculated in terms of the base.

In 45 mice injected at three dose levels of 0.55, 1.09 and 2.18 mgm per kg the LD₅₀ was 0.98 ± 0.184 mgm per kg for epinephrine. For ethylnorsuprarenin a total of 240 mice were used with results depicted in figure 1. The LD₅₀ as calculated from the Winthrop log probit paper, by a method being described (12), was 117 ± 1.04 mgm per kg. Generalizations as to the relative toxicity

² We are indebted to Drs. T. J. Becker and L. C. Miller of the Winthrop Chemical Company for the mortality data given here.

of the two compounds are not justified because of the lack of parallelism of the dosage-action curves shown in figure 1. Nevertheless at the LD_{50} level epinephrine is 119 times as toxic as ethylnorsuprarenin for acute injections. These data demonstrate a greatly enhanced margin of safety of ethylnorsuprarenin over epinephrine which would permit a much more liberal dosage schedule, if this were needed.

CIRCULATION. Intravenous injections. Thirty individuals were used for intravenous administrations. They were allowed to rest in bed until the circulation was constant as judged by repeated measurements of blood pressure and pulse rate. Then, 0.5 mgm. was injected intravenously and the measurements continued until restored to normal. The average changes are shown graphically in figure 2. The first and most pronounced effect was a sharp rise in pulse rate from 82 to 110 per minute, followed by a drop in diastolic pressure from 80 mm.

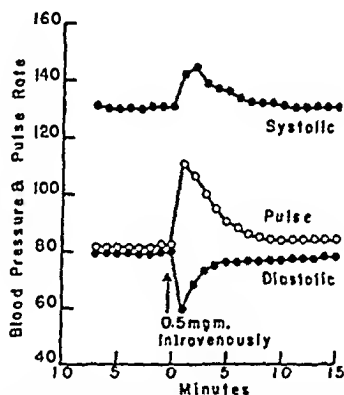


FIG. 2. AVERAGE CHANGES IN BLOOD PRESSURE AND PULSE RATE IN 30 PATIENTS INJECTED INTRAVENOUSLY WITH 0.5 MG. OF ETHYLNORSUPRARENIN

to 59 mm. and a more slowly developing systolic rise from 131 mm. to 140 mm. This resulted in a marked increase in pulse pressure from the control of 51 mm. to 83 mm. It is highly probable that the increased pulse rate and pulse pressure together indicated a considerable increase in the cardiac output. The effects were rather transient, the greater part of the response being over in 5 minutes and practically complete restoration to normal coming in about 15 minutes. Epinephrine injected intravenously raised markedly both systolic and diastolic pressures, in contrast to the relatively small increase in systolic and decrease in diastolic pressures after ethylnorsuprarenin. There was also no slowing of the pulse through reflex vagus activation from the latter.

Six of the patients in this series were hypertensives, but in them the average changes coincided, within the limits of error, with the averages of the entire series. Hence, there was no indication that they responded differently from those with normal circulation.

Intramuscular injections. In 8 patients, intramuscular injections of 1 mgm. of

ethylnorsuprarenin were made, and the same circulatory observations as for the intravenous injections. The average results are shown in figure 3. The pulse rate rose from 77 to 91 beats per minute, the change lasting 17 minutes, and possibly longer. The systolic pressure changed only negligibly, there being a small drop of 2 mm followed by a rise of 4 from the control values. The diastolic pressure however, was distinctly lowered from the control of 73 to 63 mm,

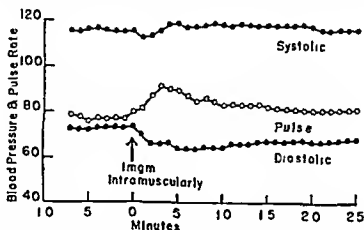


FIG 3 AVERAGE CHANGES IN BLOOD PRESSURE AND PULSE RATE IN 8 PATIENTS INJECTED INTRAMUSCULARLY WITH 1 MGm OF ETHYLNORSUPRARENIN

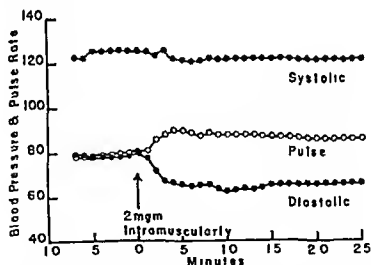


FIG 4 AVERAGE CHANGES IN BLOOD PRESSURE AND PULSE RATE IN 6 PATIENTS INJECTED INTRAMUSCULARLY WITH 2 MGm OF ETHYLNORSUPRARENIN

without complete recovery at the end of 25 minutes when the observations were discontinued. Again there were evidences of lowered peripheral resistance and increased cardiac output since there was an increased pulse rate accompanied by an increased pulse pressure.

In 6 patients a dose of 2 mgm was injected intramuscularly, with average results shown in figure 4. The systolic pressure dropped about 5 mm, while the diastolic decreased 16 mm giving a maximal increase in pulse pressure from the control of 46 to 58 mm. The pulse accelerated at the same time from 79 to

89 beats per minute. At the end of 25 minutes, the diastolic pressure and pulse had recovered only slightly, so that the response must have persisted at least more than one-half hour.

Subcutaneous injection. In one individual an attempt was made to continue the observations after a subcutaneous injection of 2 mgm. until complete recovery had occurred. The control blood pressure was 135/78 mm. with a pulse of 73 per minute. After the injection, as shown in figure 5, the pulse pressure gradually widened and the pulse rate increased slightly, until the maximum effect was present 13 minutes after the injection. At this time, the pulse pressure was 98 mm., an increase of 41 mm. over the control period. The changes persisted until the end of 37 minutes after the injection, when a gradual recovery

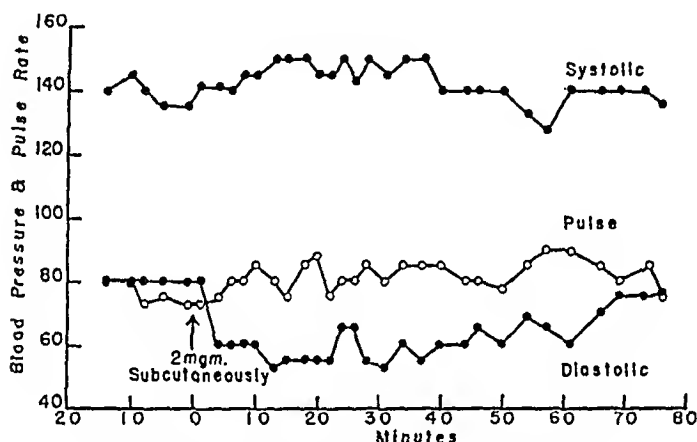


FIG. 5. CHANGES IN BLOOD PRESSURE AND PULSE RATE IN A PATIENT INJECTED SUBCUTANEOUSLY WITH 2 MG. OF ETHYLNORSUPRARENIN

towards normal began. Complete recovery required at least an hour, demonstrating a rather persistent action for an epinephrine-like compound.

Summary of circulatory changes. The striking features of these circulatory actions are a faster pulse rate and a moderate decrease of diastolic pressure, with little accompanying change in systolic pressure, except transiently after intravenous injection. Consideration of these changes and the doses used, in comparison with epinephrine, leaves no doubt that ethylnorsuprarenin possesses distinctive effects on the circulation, differing both qualitatively and quantitatively from epinephrine. The lack of violent pressor effects would, therefore, seem to afford the opportunity of using this drug for selected sympathetic actions without the usual limitations of concomitant hypertension.

EFFECTS IN ASTHMA. Ethylnorsuprarenin was used in 47 patients with allergy in which detailed records could be kept, and in a considerable number of additional patients under less favorable circumstances. The ages of the patients ranged from 3 to 64 years, the majority being adults. The clinical conditions

were the usual kind in acute allergic states, ranging from status asthmaticus, seasonal asthma and hay fever, to the milder states of specific sensitivity. The cases selected were only those whose responses to epinephrine were previously known from the effects of many previous injections and long observation. The group of cases was, therefore, not one of random selection, because of the purpose of not only determining clinical effectiveness, but also of making a direct comparison with epinephrine. Relief of the asthma was judged by the objective criteria of respiratory rate and depth, comparative length of expiration and inspiration, and disappearance of physical signs in the chest.

The doses used ranged from 0.2 to 2.0 mgm. ethylnorsuprarenin injected subcutaneously in the majority of the patients, but intramuscularly in 7 of them. The average dose was approximately 0.75 mgm., which was about 50 per cent more than was required of epinephrine in the same patients for the same degree of relief. In 46 per cent of the patients, the degree of relief was the same as that from epinephrine, in 37 per cent there was better relief than from the highest tolerated doses of epinephrine, and in the remainder (17 per cent) the epinephrine gave better relief. We interpret these results as indicating that ethylnorsuprarenin can produce at least as good relief as epinephrine, when used in appropriate doses.

The main differences in the responses were in the side effects. In 8 patients, epinephrine injections regularly caused precordial pain or discomfort. This was not experienced by any of the 8 when they were injected with equally effective doses of the new compound. Nausea and vomiting were an invariable result of epinephrine administration in 7 of the patients, but 5 of these tolerated the ethylnorsuprarenin without difficulty, and no other patients experienced nausea or vomiting after the latter drug. Tremors, nervousness and excitement were constant occurrences after epinephrine in 21 patients. This was seen in only 15 of them after ethylnorsuprarenin, and, in most of these 15, the intensity of these symptoms was less than after epinephrine.

The overall impression of clinical desirability in comparison to epinephrine was recorded in 34 patients, taking into account the degree of relief as well as the side actions. In 4 of the patients in whom epinephrine caused no particular side effects, it was preferred to ethylnorsuprarenin. In 11 there was no clear advantage in one drug over the other, while in the remaining 19, the ethylnorsuprarenin seemed the better because of causing complete or better relief with less symptoms than the epinephrine.

These results lead to the conclusion that ethylnorsuprarenin is effective in treating asthmatic states and that it will cause degrees of relief comparable to those of epinephrine when injected in somewhat higher doses. It produces its therapeutic effects without causing as many and as marked side actions as epinephrine, the relative absence of precordial pain, nausea and vomiting in susceptible patients being particularly noteworthy. Therefore, ethylnorsuprarenin would seem to be a welcome addition to the therapeutic armamentarium, having a clinical effectiveness at least as good as epinephrine, but being preferable for children because of the less severe side effects and for adults with cardio

vascular disease, or, in general in those who do not satisfactorily tolerate adequate doses of epinephrine.

CONCLUSIONS

1. The chemical compound 1-(3,4-dihydroxyphenyl)-2-amino-1-butanol hydrochloride or ethylnorsuprarenin is a sympathomimetic amine, closely resembling epinephrine in many of its actions. However, it lacks the power to raise systolic blood pressure like epinephrine, but rather lowers the diastolic pressure and increases the pulse rate. Therefore, it causes an increase in pulse pressure, and presumably in the volume flow of blood, without a proportionate rise in cardiac work.

2. In animals, ethylnorsuprarenin is approximately one-one hundred and twentieth as toxic as epinephrine for fatal effects intravenously, and does not cause excitation of the central nervous system.

3. In patients, ethylnorsuprarenin may be injected subcutaneously, intramuscularly or intravenously in doses of from 0.5 to 2.0 mgm., with typical effects which appear in from 1 to 5 minutes and persist for from 20 minutes to an hour, depending on the dose and route of administration.

4. It is effective in the relief of acute asthmatic attacks, giving relief equal to that of epinephrine in doses about one-half larger. This relief is accompanied by fewer and less marked subjective side effects, such as precordial pain, nausea and vomiting, excitation, etc., than might be experienced by the same subjects after equivalent doses of epinephrine.

5. Accordingly, ethylnorsuprarenin would appear to be useful in the treatment of asthma, and possibly preferable to epinephrine for those patients in whom epinephrine administration is accompanied by undesirable side-actions. Further clinical trials appear to be justified along these and other lines.

REFERENCES

1. TAINTER, M. L.: Comparative actions of sympathomimetic compounds: phenyl and substituted phenyl derivatives. *Non-phenylic ring compounds, and aliphatic amines*. Archives Internationales de Pharmacodynamie et de Therapie, 46: 192, 1933.
2. TAINTER, M. L., PEDDEN, J. R., AND JAMES, M.: Comparative actions of sympathomimetic compounds: bronchodilator actions in perfused guinea pig lungs. *This Journal*, 51: 371, 1934.
3. PEDDEN, J. R., TAINTER, M. L., AND CAMERON, W. M.: Comparative actions of sympathomimetic compounds: bronchodilator actions in experimental bronchial spasm of parasympathetic origin. *This Journal*, 55: 242, 1935.
4. CAMERON, W. M., AND TAINTER, M. L.: Comparative actions of sympathomimetic compounds: bronchodilator actions in bronchial spasm induced by histamine. *This Journal*, 57: 152, 1936.
5. CAMERON, W. M., CRISMON, J. M., WHITESSELL, L. J., AND TAINTER, M. L.: Analysis of the circulatory actions of ethylnorsuprarenin. *This Journal*, 62: 318, 1938.
6. CAMERON, W. M., WHITESSELL, L. J., CRISMON, J. M., AND TAINTER, M. L.: Further evidences on the nature of the vasomotor actions of ethylnorsuprarenin. *This Journal* 63: 340, 1938.
7. TAINTER, M. L., FOOTER, A. W., AND HANZLIK, H.: Sympathomimetic stimulants in acute circulatory failure of phenol shock. *Am. J. Med. Sci.*, 197: 796, 1939.

- 8 CRISMON, C A , AND TAINTER, M L Comparative pressor efficiency of sympathomimetic amines in the normal state and in decerebrate shock *THIS JOURNAL*, 66: 146, 1939
- 9 TAINTER, M L , WHITSELL, L I , AND DILLE, J M The analeptic potency of sympathomimetic amines *THIS JOURNAL*, 67: 56, 1939
- 10 MORTON, M C , AND TAINTER, M L Effects of sympathomimetic amines on perfused blood vessels *J Physiol*, 98: 263, 1940
- 11 SCHULTE, J W , REIF, E C , BACHER, J A , LAWRENCE, W S , AND TAINTER, M L Further study of central stimulation from sympathomimetic amines *THIS JOURNAL*, 71: 62, 1941
- 12 MILLER, LLOYD C , AND TAINTER, M L Estimation of the ED_{50} and its 'standard error on log probit graph paper' (in press)

THE TOXICITY AND TRYPANOCIDAL ACTIVITY OF p-SULFON-AMIDOPHENYLARSONIC ACID AND CERTAIN OF ITS DERIVATIVES¹

E. LEONG WAY AND L. K. CHAN

From the Department of Pharmacology, The George Washington University, School of Medicine, Washington, D. C., and the Department of Pharmacology, University of California, School of Medicine, San Francisco, Calif.

Received for publication May 4, 1944

The numerous applications of sulfanilamide and its derivatives to a great variety of infections have led certain investigators to believe that related aromatic arsenic compounds containing the sulfonamide or substituted sulfonamide groups might possibly yield therapeutically active agents. Only a few phenylarsonic acids containing the sulfonamido group have been described and comparatively little is known as to the pharmacological effect of such substitution on the aromatic group. Gough and King (1) first prepared p-sulfonamidophenylarsonic acid and its oxide, and on subsequent pharmacological study, found that the compounds exhibited trypanocidal activity. These favorable findings led Oneto and Way (2, 3, 4) to synthesize some N substituted derivatives of p-sulfonamidophenylarsonic acid; they also prepared the latter compound but by a different method. The present communication is essentially a report of studies on the toxicity and trypanocidal activity of the above compounds, namely, p-sulfonamidophenylarsonic acid and certain of its derivatives.

METHOD. Albino mice weighing approximately 20 grams were used for determining the toxicity and trypanocidal activity of the compounds. The latter effect was obtained by employing *T. equiperdum*, as well as *T. brucei*, inoculations being made intraperitoneally with saline suspensions (0.9%) of the organisms. The compounds were also injected intraperitoneally, the concentration of a given dose being so adjusted that not more than 1 cc. and not less than 0.25 cc. was used for each animal. In two instances where the compounds were found inactive by the intraperitoneal route, they were also administered perorally. The observation period for toxicity was 72 hours. Infected mice were not treated until a count showed approximately 100,000 trypanosomes per cu. mm. of blood. A compound was considered effective if the blood stream was free from organisms for 72 hours, and curative, if free for 21 days. The maximum tolerated dose, M.T.D.; minimum effective dose, M.E.D.; and minimum curative dose, M.C.D., were arbitrarily set at the dose yielding approximately 80% effect, i.e., M.T.D., etc.

RESULTS. The results are shown in tables 1 to 3 inclusive. As can be ascertained from table 3, a wide toxicity range was obtained with the different derivatives. The tolerated dose varied in the pentavalent arsenic compounds from 10 mgm./kgm. (p-arsono-N-benzenesulfonylpiperidide) to 2500 mgm./kgm. (p-arsono-N-(p-carboxyphenyl)benzenesulfonamide), and from 3 mgm./kgm.

¹ Presented before the Division of Medicinal Chemistry of The American Chemical Society at Cleveland, Ohio, April 6, 1944.

(p arsenoso N phenylbenzenesulfonamide) to 40 mgm /kgm (p arsenoso N (p carboxy phenyl) benzenesulfonamide monohydrate) for the trivalent derivatives

With p sulfonamidophenylarsonic acid the toxic symptoms sometimes produced by pentavalent arsenic compounds in mice were present, that is, persistent tremors and gyrations which were apparently similar to the dancing mice"

TABLE 1
Results on p sulfonamidophenylarsonic acid

TOXICITY		TRYPANOCIDAL ACTIVITY	
Dose	Result	Dose	Result
mgm /kgm	deaths/no of mice	mgm /kgm	cures/no mice
2000	8/15	1000	2/2
1900	1/10	700	6/8
1800	1/13	500	3/8
1700	2/17	300	1/5
1600	4/21	200	0/4
1500	1/6	100	0/2
1250	0/1		
M T D = 1900 mgm /kgm		M C D = 700 mgm /kgm	

$$\text{Chemotherapeutic Index} = \frac{\text{M T D}}{\text{M C D}} = 2.7$$

TABUL 2
Results on propamidine

TOXICITY		TRYPANOCIDAL ACTIVITY	
Dose	Result	Dose	Result
mgm /kgm	deaths/no of mice	mgm /kgm	cures/no mice
50	4/7	10	4/4
40	0/10	7	5/5
30	2/9	5	9/11
		2*	0/5
M T D = 40 mgm /kgm		M C D = 5 mgm /kgm	

$$\text{Chemotherapeutic Index} = \frac{\text{M T D}}{\text{M C D}} = 8.0$$

* Effective but not curative

described by Ehrlich Gough and King (1) reported that this compound was tolerated in doses greater than 1500 mgm /kgm and was curative for 16 days at 1000 mgm /kgm we find it tolerated at 1900 mgm /kgm and curative for 21 days at 700 mgm /kgm

We find propamidine (4,4'-diaminodiphenoxypropane dihydrochloride) very effective in establishing a cure in experimental trypanosomiasis. The compound was tolerated at 40 mgm /kgm and curative at 5 mgm /kgm. The results

coincide with those reported by Ashley and colleagues (5) although we administered the drug intraperitoneally instead of intravenously; Lourie and Yorke

TABLE 3

Toxicity and trypanocidal activity of *p*-sulfonamidophenylarsonic acid, its derivatives, and of propamidine in mice infected with *T. equiperdum*

PENTAVALENT COMPOUNDS	FORMULA	M.W.	As %	M.T.D.	M.C.D.*	INDEX
1. <i>p</i> -arsonobenzenesulfonamide* (<i>p</i> -sulfonamidophenylarsonic acid)...	C ₆ H ₅ O ₂ NSAs	187.09	26.66	1900	700 ^d	2.7
2. <i>p</i> -arsono- <i>N</i> -dimethylbenzenesulfonamide ^b . .	C ₈ H ₁₂ O ₄ NSAs	309.15	24.21	80	Inactive	—
3. <i>p</i> -arsono- <i>N</i> -(<i>p</i> -carboxyphenyl) benzenesulfonamide ^b	C ₁₃ H ₁₂ O ₇ NSAs	401.20	18.67	2500	Inactive ^c	—
4. <i>p</i> -arsono- <i>N</i> -(<i>p</i> -sulfonamidophenyl) benzenesulfonamide ^b	C ₁₂ H ₁₀ O ₇ N ₂ S ₂ As	436.27	17.18	500	Inactive ^f	—
5. <i>p</i> -arsono- <i>N</i> -benzenesulfonylpiperidide ^c .	C ₁₁ H ₁₆ O ₆ NSAs	349.22	21.45	10	Inactive	—
6. <i>p</i> -arsono- <i>N</i> -benzenesulfonylmorpholide ^c	C ₁₀ H ₁₄ O ₆ NSAs.....	351.19	21.33	100	Inactive	—
TERVALENT COMPOUNDS						
7. <i>p</i> -arsono- <i>N</i> -phenylbenzenesulfonamide ^b .	C ₁₂ H ₁₀ O ₃ NSAs	323.18	23.18	3	Inactive	—
8. <i>p</i> -arsono- <i>N</i> -(<i>p</i> -carboxyphenyl) benzenesulfonamide monohydrate ^b	C ₁₃ H ₁₂ O ₆ NSAs	385.19	19.45	40	Inactive	—
9. <i>p</i> -arsonobenzenesulfonylmorpholide ^c	C ₁₀ H ₁₂ O ₄ NSAs	317.18	23.62	4	Inactive	—
10. 4:4'-diamidinodiphenoxypropane dihydrochloride (propamidine) ..	C ₁₇ H ₂₀ O ₂ N ₄ ·2HCl	385.29	—	40	5	8.0

* Oneto, J. F., and Way, E. L., J. Am. Chem. Soc., 61: 2105, 1939.

^b Oneto, J. F., and Way, E. L., Ibid., 63: 762, 1941.

^c Way, E. L., and Oneto, J. F., Ibid., 64: 1287, 1942.

^d Effective but not curative at 1000 mgm./kgm. perorally.

^e Inactive 4000 mgm./kgm. perorally.

^f Inactive 3000 mgm./kgm. perorally.


* All compounds inactive against *T. equiperdum* were also inactive against *T. brucei* at $\frac{1}{2}$ the tolerated dose.

(6) using intraperitoneal administration, reported a tolerated dose of 50 mgm./kgm. and a curative dose of 2.5–5.0 mgm./kgm.

More complete statistical studies on propamidine and *p*-sulfonamidophenylarsonic acid were not made inasmuch as none of the *N* substituted derivatives of the latter compound showed trypanocidal activity. All compounds found

inactive against *T. equiperdum* were also inactive against *T. brucei* when administered in single doses of approximately one half their respective tolerated dosage



BIOCHEMOPHLOGY p Sulfonamidophenylarsonic acid, , repre



sents a distinctly different type of an aromatic arsenic compound with tripanocidal properties, the free acid itself, p sulfophenylarsonic acid, is inactive (1)

Gough and King (1) have shown that the amide group has an important influence in converting tripanocidally inactive carboxylic and sulfonic acids of aromatic arsenic compounds into substances of marked activity. It follows then that in order for an amide or any N substituted derivatives of phenyl arsonic acid to be active, the integrity of the amide group must be maintained in the host and the parasite because the hypothetical products of amide hydrolysis the carboxy or sulfophenylarsonic acid and its respective amine, are virtually inactive. Although the activity of the amide compounds is dependent upon the integrity of the amide linkage, the presence of an amide group does not necessarily ensure that a compound will be active. This is apparent from our results, none of our derivatives were tripanocidal. These conditions should hold also for the amides of the reduced phenyl arsonic acids, i.e., arsine oxides.

Inasmuch as none of our N substituted sulfonamide derivatives exhibited tripanocidal activity, one might be tempted to postulate that the compounds were inactivated by the hydrolysis of the sulfonamido linkage, such a reaction occurs readily *in vitro*. However it appears improbable that the reaction occurs in the host because the high toxicity of certain derivatives (e.g., tolerated dose of p arsono benzenesulfonylpiperidide 10 mgm/kgm, p arsono N dimethyl benzenesulfonamide, 80 mgm/kgm etc.) cannot be explained on this basis the products of hydrolysis of the amides p sulfophenylarsonic acid [tolerated dose, 500 mgm/kgm (1)] and the respective amines should not be toxic in the small doses that were administered. Therefore, in order for the substituted sulfonamido derivatives to be toxic in lower doses than p sulfophenylarsonic acid, the entity of the sulfonamido group must have been maintained in the host.

It is true that certain compounds were tolerated in much higher doses than p sulfophenylarsonic acid e.g. tolerated doses of p arsono N (p carboxy phenyl) benzenesulfonamide 2500 mgm/kgm, p arsono benzene sulfonamide 1900 mgm/kgm. This can be explained in two ways either the compounds *per se* are non toxic to the host or they are hydrolyzed very slowly to liberate the more toxic free sulfo acid (tolerated dose 500 mgm/kgm) which can then be rapidly eliminated before a toxic level is reached. We believe the latter explanation to be unlikely because we have not encountered any report in the literature that a sulfonamido linkage can be cleaved *in vivo* to yield a free sulfo acid and an amine although as we have already indicated such a reaction can occur *in vitro*. In the voluminous reports on the excretion of sulfonamide and its derivatives,

none of the compounds have been reported to be eliminated as sulfo acids, indicating perhaps that no enzyme is present in the body for such function.

Gough and King (1) have attempted to explain why an amide group can convert inactive p-arsenophenylcarboxylic and sulfonic acids into trypanocidally active compounds. They attributed the increase in activity to the fact that the compounds with an amide group are excreted at a lower rate than their free carboxylic or sulfonic acids because upon reduction of the amido derivatives in the body, they will exist in a colloidal state; whereas, those containing the free acid grouping will exist as crystalloids, and therefore, will be eliminated more rapidly. We agree that the free acids may exist as crystalloids and be excreted more easily, but we do not think the state of the amido arsine oxides is necessarily a colloidal one because arsine oxides are active in such small amounts, and when in solution generally do not precipitate out as readily as the very insoluble arspenamines upon changes in pH; then too, the protective colloids in the body will tend to aid in holding the small quantities of arsine oxide present in solution. For example, oxophenarsine hydrochloride (mapharsen) can be precipitated with dilute alkali *in vitro*, only from a concentrated solution of the drug. Moreover, most investigators believe it acts as a crystalloid *in vivo*, and Roth and Creswell (7) have presented evidence that oxophenarsine hydrochloride diffuses more readily and to a much greater degree through gelatin than do the semi-colloidal arspenamines.

Gough and King (1) have also shown that the trypanocidal activity of their substituted amide derivatives decreased with an increase in the size of the substituting alkyl group or with the introduction of more than one alkyl group. They explained the diminished therapeutic effects by saying, "the introduction of weakly positive groups will cause a drift of electrons towards the arsenic atom relative to the effect of the unsubstituted amide group, and this will be reflected in a diminished ease of reduction of arsonic acids (since the oxygen will be more firmly held) and in a decreased tendency to coordinate with hydroxyl (since the arsenic atom has become more negative with resultant increased difficulty of hydrolysis)." It seems rather doubtful that such weakly electropositive groups are significant in influencing the ease of reduction and hydrolysis. According to their theory electronegative groups should then show trypanocidal activity inasmuch as their effects are opposite to that of the electropositive alkyl groups; we found that p-arsenos-N-phenylbenzenesulfonamide, which contains the negative phenyl group, to be inactive. Eagle, Hogan, Doak, and Steinman (8) have reported also that p-arsenos-N-phenylbenzamide is much less active than p-arsenosobenzamide as a spirocheticide.

The compounds which we investigated represent a good portion of that particular type which has been reported, but the possibilities of preparing more derivatives are unlimited. We hesitate, therefore, to advance any general rules in regards to the effect of substitution on the nitrogen of the sulfonamide group. However, the indications seem to be that *in vivo* alkyl groups increase toxicity and decrease trypanocidal activity. In addition, the use of relatively toxic amines such as piperidine, morpholine, and aniline to form their respective

amides produces the more toxic derivatives. On the other hand condensing with p aminobenzoic acid and sulfanilamide results in less toxic compounds being formed. There seems to be very little relationship between toxicity and total arsenic content. These views are substantiated in part by the findings of Eagle, *et al* (8) who found that the treponemicidal activity of p arsenosobenzenesulfonamide was decreased and the toxicity was increased by N substitution of alkyl groups. They also found, however, that certain alcohol radicals ($-\text{C}_2\text{H}_4\text{OH}$, $-\text{CH}_2-\text{CHOH}-\text{CH}_2\text{OH}$, etc.) and certain other substituents did not materially affect the ratio of treponemicidal activity to toxicity (*in vitro* studies and calculated on a mole basis), and some increased the ratio slightly.

Further studies are warranted along these lines to abet the search for a new therapeutic agent, and to aid in comprehending the biochemorphology of such derivatives.

ACKNOWLEDGMENTS We are grateful to Professor G. B. Roth for his timely advice and the supply of propamidine to Mr. M. T. S. Proby for the *T. equiperdum*, to Professor J. F. Oneto for the arsenic compounds, to Professor W. D. Kumler for his opinions, and to Messrs. Rollan Swanson and Walter Schivler for their technical assistance.

SUMMARY

1 The toxicity of p sulfonamidophenylarsonic acid, its derivatives and of propamidine are reported.

2 The tolerated dose of p sulfonamidophenylarsonic acid in mice was found to be 1900 mgm /kgm and the curative dose 700 mgm /kgm.

3 Propamidine was found to be tolerated at 40 mgm /kgm and curative at 5 mgm /kgm.

4 None of the N substituted derivatives of p sulfonamidophenylarsonic acid showed trypanocidal activity.

5 A discussion of the biochemorphology of the compounds is included.

BIBLIOGRAPHY

- 1 GOUGH G. A. C. AND KING H. J. *Chem Soc* 669, 1930
- 2 ONETO J. F. WAY, E. L. *J Am Chem Soc* 61 2105 1939
- 3 ONETO J. F. WAY, E. L. *Ibid* 63 762 1941
- 4 WAY E. L. ONETO J. F. *Ibid* 64 1287 1942
- 5 ASHLEY J. N. BARBER H. F. EWINS A. J. NEWBERRY, G., AND SELF, A. B. H., *J Chem Soc* 103 1942
- 6 LOURIE E. M. AND YORKE W. *Ann Trop Med Parasit* 33 289 1939
- 7 ROTH G. B. AND CRESWELL G. W. *Med Ann of D C* 6 195 1937
- 8 EAGLE H. HOGAN R. B. DOAK G. O. AND STEINMAN H. G. *J Am Chem Soc*, 65 1236 1943.

THE ACUTE TOXICITY FOR MICE OF "MAPHARSEN" AND SODIUM SULFATHIAZOLE ADMINISTERED SEPARATELY AND IN COMBINATION¹

ELIZABETH M. CRANSTON, WILLIAM G. CLARK AND ERNEST A. STRAKOSCH

From the Departments of Pharmacology and Zoology of the University of Minnesota, Minneapolis, Minnesota and the Department of Dermatology, College of Medicine, University of Illinois, Chicago, Illinois

Received for publication May 17, 1944

The same individual sometimes requires treatment for both syphilis and gonorrhea. Since certain drug combinations are frequently used in such cases, it is of interest to know whether the antisiphilitic arsenical compounds and the antigonorrheal sulfonamides, given in combination, are synergistic, additive, antagonistic or without effect on one another as regards toxicity. "Mapharsen" (3-amino-4-hydroxyphenylarsine oxide hydrochloride) and sodium sulfathiazole (sodium salt of 2-sulfanilamido-thiazole) were selected as commonly used representatives of these two groups of drugs and their separate and combined toxicities for mice were studied.

METHODS. Test animals were young male and female mice of Strain A (1) weighing between 14 and 20 grams. Studies on the separate toxicity of "Mapharsen" and sodium sulfathiazole were run in parallel over a period of about one month in groups of 10 or 20 animals each. The combined toxicity experiments were conducted over a period of about six weeks, again in groups of 10 or 20 animals each. Thus the data in the tables presented represent a summation of the results obtained from various experiments. The work was carried out during the winter months and the animals were kept at room temperature, about 78°F.

Sodium sulfathiazole (Merck)² was administered as a 10 per cent solution in distilled water. "Mapharsen" (Parke, Davis and Co.)³ was administered as a 0.3 per cent solution in distilled water. Solutions of the latter were prepared from ampules of the drug as supplied for clinical use. Each ampule contained sufficient sodium carbonate to form the sodium salt and the entire contents of each ampule were used to prepare the solution in order to avoid errors due to any uneven distribution of the components present in dry form. Both drug solutions were injected not later than 90 minutes after the time of preparation. A total of 780 animals was used. The LD_{50} of a single intraperitoneal injection of sodium sulfathiazole was determined on 170 mice and the LD_{50} of a single intraperitoneal injection of "Mapharsen" was determined on 210 mice. To determine the combined toxicity of the two drugs, fractions of the previously determined LD_{50} of each were administered intraperitoneally to 370 mice. The sodium sulfathiazole was injected ten minutes prior to the injection of "Mapharsen". The data in the tables below include all deaths occurring within one week of injection. Calculations of the LD_{50} were made according to the method of Litchfield and Fertig (2), from dosage-effect curves plotted from probits of the per cent mortality and logarithms of the doses.

¹ This work was supported by a grant from Parke, Davis and Co., Detroit, Mich.

² Generously donated by Merck and Co., Inc., Rahway, N. J.

³ Generously donated by Parke, Davis and Co., Detroit, Mich.

RESULTS The data obtained on the acute toxicity of "Mapharsen" alone are presented in table 1, from which the LD_{50} of "Mapharsen" was calculated to be 34 ± 0.5 mgm per kgm

All mice were kept for 30 days. A few additional deaths occurred between 8 and 29 days. If these were included in the calculations, the LD_{50} would be 30.6 mgm per kgm

TABLE 1

Data for the determination of the LD_{50} of "Mapharsen" intraperitoneally in mice

DOSE	NO MICE	NO DIED	MORTALITY	AV TIME OF DEATH
mgm /kgm			%	Hours
15	10	0	0	
20	10	0	0	
25	30	1	3.3	144
30	30	3	26.6	74
32.5	30	4	13.3	89
35	30	8	26.6	85
37.6	30	20	66.6	62
40	20	19	95	47
45	10	10	100	31
50	10	10	100	4

TABLE 2

Data for the determination of the LD_{50} of sodium sulfathiazole intraperitoneally in mice

DOSE	NO MICE	NO DIED	MORTALITY	AV TIME OF DEATH
gram /kgm			%	Hours
0.8	10	0	0	
1.0	10	0	0	
1.2	30	9	30	35
1.3	30	10	33.3	35
1.4	30	20	66.6	22
1.5	30	25	83.3	23
1.6	30	29	96.6	18

The data obtained on the acute toxicity of sodium sulfathiazole alone are presented in table 2, from which the LD_{50} was calculated to be 1.32 ± 0.02 grams per kgm

Intraperitoneal injections of 1.9 per cent sodium carbonate, which is slightly more than equimolecular (1.7 per cent) to a 10 per cent solution of sodium sulfathiazole with respect to sodium, were made in 30 mice in doses corresponding to 1.4 grams per kgm of sodium sulfathiazole. There were two deaths in the group one in two days and one in five days. That these were alkalosis deaths seems doubtful. The only other symptom of toxicity in any of the animals was a slight increase in irritability within the first 30 minutes following injection.

The slopes of the dosage effect curves drawn for "Mapharsen" and sodium

sulfathiazole were similar, the slope constants being 19 ± 1.28 and 20 ± 2.43 respectively. Accordingly the change in dosage required to alter the response by one probit was 13 per cent for "Mapharsen" and 12 per cent for sodium sulfathiazole. Since the slope constants were not significantly different, identical fractions of the LD_{50} of each drug could be used in determining the combined toxicities.

The results obtained for "Mapharsen" and sodium sulfathiazole in combination appear in table 3. Calculations from these data showed the LD_{50} of the combination to be 65 per cent ± 1.5 per cent of the LD_{50} of each drug alone.

All mice were kept for 30 days. A few additional deaths occurred between 8 and 24 days. If these were included the LD_{50} would be 63 per cent of the LD_{50} of each drug alone. However, if one includes all deaths within 30 days, then the smaller LD_{50} of "Mapharsen" (30.6 mgm. per kgm.) should be used, which would theoretically, at least, increase the LD_{50} of the combination.

TABLE 3

Data for the determination of the LD_{50} of "Mapharsen" plus sodium sulfathiazole intraperitoneally in mice

DOSE AS PER CENT OF LD_{50} OF EACH DRUG	NO. MICE	NO. DIED	MORTALITY	AV. TIME OF DEATH
			%	hours
20	10	0	0	
30	10	0	0	
40	60	4	6.6	70.2
50	60	5	8.3	91.8
60	60	17	28.3	57.3
70	60	28	46.6	19.6
80	60	50	83.3	19.6
90	30	30	100	13.1
100	20	20	100	2.5

DISCUSSION. In a personal communication the manufacturers (3) give the LD_{50} of "Mapharsen" in mice as 20 mgm. per kgm. intravenously and 25.0-27.5 mgm. per kgm. subcutaneously. The LD_{50} of sodium sulfathiazole for mice has been reported to be 1.45 grams per kgm. subcutaneously by Van Dyke, et al. (4), 1.32 grams per kgm. subcutaneously by Walker and Van Dyke (5), 1.95 grams per kgm. "parenterally" by Long, et al. (6), 0.708 grams per kgm. intravenously by Powell and Chen (7), and from 0.845 to 1.244 grams per kgm. intravenously, depending upon environmental temperature, by Chen, et al. (8).

The toxicity of sodium sulfathiazole intraperitoneally as reported in this paper is approximately the same as values reported by others for subcutaneous injections, and somewhat lower than the results with intravenous injections. The toxicity of "Mapharsen" intraperitoneally as reported here is lower than that found by others (3) using subcutaneous as well as intravenous injections.

Although the combination of both drugs was found to be more toxic than either alone (the LD_{50} of the combination being 65 per cent of the LD_{50} of each

alone), the combined toxicity was not synergistic since if that had been the case the LD_{50} of the drugs together should have been less than 50 per cent of the LD_{50} of each alone. The results obtained indicate that the toxic effects are additive but are less than algebraic summation.

SUMMARY

1 Under the experimental conditions described, the LD_{50} of an intraperitoneally administered 0.3% aqueous solution of "Mapharsen" (3-amino-4-hydroxyphenylarsine oxide hydrochloride, administered as the sodium salt) in young "Strain A" mice, was found to be 34.0 ± 0.5 mgm per kg.

2 The LD_{50} of a 10% aqueous solution of sodium sulfathiazole administered similarly, was found to be 1.32 ± 0.02 grams per kgm.

3 The combined toxicity of "Mapharsen" and sodium sulfathiazole was found to be greater than the toxicity of either drug alone, since the LD_{50} of the combination was 65 per cent of the LD_{50} of each drug alone.

REFERENCES

- 1 GÜNEBERG, H., 'The Genetics of the Mouse' Cambridge U. Press, 1943, p. 319.
- 2 ITCHINFIELD, J. T. JR., AND FERTIG, J. W., Bull. Johns Hopkins Hosp., 69: 276, 1941.
- 3 Parke, Davis and Co., Detroit, Mich., Personal communication.
- 4 VAN DYKE, H. B., GRLEY, R. O., RAKE, G., AND MCKEE, C. M., Proc. Soc. Exp. Biol. and Med., 42: 410, 1939.
- 5 WALKER, H. A., AND VAN DYKE, H. B., This Journal, 71: 138, 1941.
- 6 LONG, P. H., HAYLAND, J. W., AND EDWARDS, L. B., Proc. Soc. Exp. Biol. and Med., 43: 328, 1940.
- 7 POWELL, H. M., AND CHEN, K. K., J. Ind. State Med. Assoc., 33: 503, 1940.
- 8 CHEN, K. K., ANDERSON, R. C., STEIDT, F. A., AND MILLS, C. A., This Journal, 79: 127, 1943.

RELATION OF THE INTENSITY OF THE MORPHINE ABSTINENCE SYNDROME TO DOSAGE

HOWARD L. ANDREWS¹ AND C. K. HIMMELSBACH

The Research Division, The U. S. Public Health Service Hospital, Lexington, Kentucky

Received for publication May 18, 1944

The literature on the treatment of the morphine abstinence syndrome is very extensive, and ranges from quantitative studies to purely descriptive discussions. Previous reports from this Laboratory (1, 2, 3) have emphasized the necessity for: 1) establishment of the fact that a patient has physical dependence prior to study or treatment; 2) rigid control over experimental conditions; 3) evaluation of the intensity of this syndrome in an objective quantitative manner; and 4) adequate untreated controls. The present communication describes an attempt to determine the relationship between the abstinence syndrome intensity (A.S.I.) and stabilization dose.² The establishment of such a relationship should aid in understanding the fundamental nature of the syndrome and should facilitate studies of withdrawal treatments.

Since 1935, patients admitted to this hospital for treatment of active addiction have required progressively smaller stabilization doses of morphine (fig. 1), and the more recent abstinence syndromes have been less severe. Addicts attribute this to the fact that opiates are now more difficult to obtain in large quantity and that illicit drugs are of poorer quality than formerly. A comparison of the data on 65 patients studied in 1935-36 with those of 41 patients under observation in 1939-40 showed that the A.S.I. of the latter group was appreciably less than that of the former. It has not been possible to correlate this difference with age, blood pressure, or any other group difference except dosage. Since that time data have been collected on sufficient additional patients and over a dose range wide enough to show that the A.S.I. bears a sufficiently close relation to dose to be useful.

METHODS. Data on the abstinence syndromes of 587 addict patients studied between September 1935 and January 1944 were available for this analysis. The presence of valid physical dependence on an opiate had been established in each patient. All were studied on a ward devoted exclusively to research on drug addiction, under the constant supervision of selected personnel especially trained in maintaining rigidly controlled conditions and in the recognition of signs of withdrawal. Each patient was stabilized for at least one week on the minimal amount of morphine required to prevent signs of abstinence. Following withdrawal intensities of the abstinence syndromes were evaluated by the point system (table 1).

Data were retained for analysis on those patients in whom withdrawal had

¹ Present address: National Institute of Health, Bethesda, Maryland.

² By stabilization dose we mean the minimal amount of morphine sulfate per day which will prevent signs of abstinence.

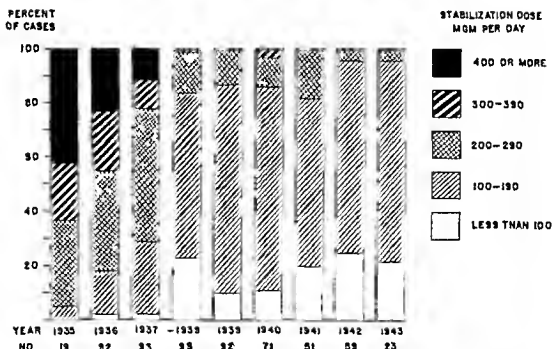


FIG 1 THIS PLOT SHOWS THE STEADY DECLINE IN STABILIZATION DOSAGES REQUIRED IN RECENT YEARS. A CORRESPONDING DECREASE HAS BEEN OBSERVED IN THE ASI.

TABLE I

Point system for measuring the abstinence syndrome intensity by the day (D) or by the hour (H)

SIGNS	(D) BY DAY		(H) BY HOUR	
	Points	Limit	Points	Limit
Yawning	1	1	1	1
Irritability	1	1	1	1
Rhinorrhoea	1	1	1	1
Perspiration	1	1	1	1
Mydriasis	3	3	3	3
Tremor	3	3	3	3
Gooseflesh	3	3	3	3
Anorexia 40% decrease in calorie intake	3	3		
Restlessness	5	5	5	5
Facies (crack spell)	5		5	5
Fever (for each 0.1°C rise over mean stabilization level)	1		1	10
Hyperpnoea (for each resp./min. over stabilization level)	1		1	10
Rise in A.M. systolic B.P. (for each 2 mm Hg over mean stabilization level)	1	15	1	10
Weight loss (for each lb. from last A.M. of stabilization)	1			

Total abstinence syndrome intensity per day or per hour is the sum of the points scored in the (D) or (H) columns respectively, with due attention to the limits.

been abrupt and complete; who were under study for at least five days of abstinence; and had served only as controls, as subjects for substitution studies of similar opiates, or as subjects for studies of withdrawal treatments found to be ineffective. The substituted opiates were: dihydromorphine, desomorphine, heroin, dihydro-heroin, alpha-isomorphine. The withdrawal treatments were: perparin, rossium, euphyllin, insulin, thiamine, pyridoxine, and pyrahexyl. Physical dependence on morphine was reproduced for experimental purposes in twenty instances. Observation showed that these patients were to all intents and purposes "stable" on the dosage given.

TABLE 2

TYPES OF STUDY	A	B	C	D	E	F	G	H	TOTALS
Morphine only..	4	7	9	1	4	2			27
Substitutions									
H2M ..		2	1	3	1				7
deso-M. .			1	3	1				5
heroin .			1	3	1				5
H2 heroin	1		4						5
alpha-iso M	1	1	3	3	1				9
H2-alpha-iso-M..			2	2	3	1			8
Treatments									
Perparin	2		1	4	1				8
Rossium . .	4	4	6	1					15
Euphyllin			1						1
insulin	1								1
thiamine			1		4				5
pyridoxine					2	1			3
pyrahexyl			1	1	1	4	1		8
Readdiction						5	10	5	20
Totals .	13	14	31	21	19	13	11	5	127

The data on 127 patients meeting these criteria were then divided into eight groups according to daily stabilization dosage. The arbitrary groupings were:

Group	Dose Range (mg morphine)	Average Dose (mg morphine)
A	400 to 500	407
B	300 to 350	302
C	200 to 280	212
D	150 to 180	160
E	100 to 140	118
F	75 to 90	80
G	60	60
H	40	40

The number of patients in each group and the studies carried out were as shown in table 2.

TREATMENT OF DATA The average ASI per day was determined for each group for the first seven days of abstinence. The plotted points were connected by straight lines and the area under each curve was measured with a planimeter. These areas were converted into point-days and then became measures of the total abstinence syndrome for seven days. This is called T.A.S.-7.

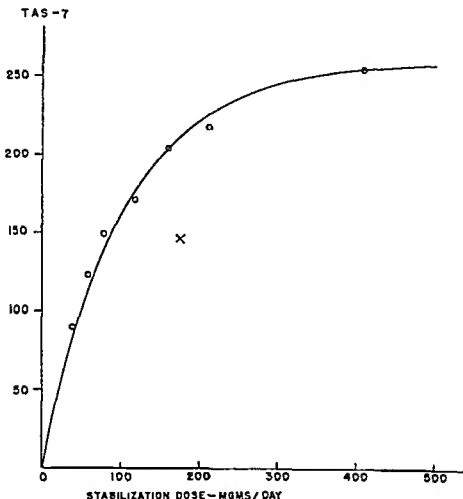


FIG. 2. T.A.S.-7 values plotted against dose. The points fell rather smoothly indicating a functional relationship between the two variables. This relationship was determined by plotting various simple functions of the variables until a linear plot was obtained. It was immediately obvious that a good fit could not be obtained with an algebraic polynomial unless an excessive number of constants were used.

When the T.A.S.-7 values were plotted against dose (fig. 2) the points fell rather smoothly indicating a functional relationship between the two variables. This relationship was determined by plotting various simple functions of the variables until a linear plot was obtained. It was immediately obvious that a good fit could not be obtained with an algebraic polynomial unless an excessive number of constants were used.

If a relationship of the form

$$y = a(1 - e^{-bx})$$

is assumed, where $y = \text{T.A.S.-7}$, $x = \text{dose}$, and a and b are constants to be determined, a plot of $\ln(a - y)$ against x should be linear. This is indeed the case, and by a suitable choice of a it is possible to determine b from the slope of the line. In determining b the best line was drawn by inspection, since the data did not seem to warrant treatment by least squares. With the constants so chosen the function becomes

$$y = 260(1 - e^{-0.0023x})$$

The smooth curve in fig. 2 is a plot of this equation with the experimental values shown as open circles. The data were also analyzed on a mg./kg. basis, the points fitting the curve about as well as those shown.

DISCUSSION. When a mathematical analysis of biological data is made care must be taken to avoid interpreting the results as if they were as exact as the mathematics employed, since even the most rigorous treatment can yield results only as good as the basic data. This fact was brought out when an attempt was made to obtain the T.A.S.-7 dose relationship using a smaller range of dosage than that described here. In that case almost equally good fits were obtained with equations of quite different character. With the present dosage range of over 10 to 1 it was possible to decide with some certainty between the various functions.

It is of importance that there is a comparatively simple relation between the T.A.S.-7 and stabilization dose. With such a relation it should be possible to predict with some accuracy the anticipated abstinence syndrome of a group of patients withdrawn from a known stabilization dose. This should help in evaluating withdrawal treatments, the effect of one treatment being shown by point "x" in fig. 2. This represents the T.A.S.-7 of a group of nine patients, with an average stabilization dose of 176 mg. (range 100-280), that received a rapid reduction treatment as described in a previous report (2). Since it is not always possible to obtain a control group with a dosage exactly equal to that of the study group, with an established T.A.S.-7 dose relationship it should be possible to correct for dose differences, and in some cases controls might be eliminated.

The form of the function obtained has several important theoretical implications. There is considerable evidence that the process of forming physical dependence involves a readjustment of autonomic equilibrium to accommodate to the effect of morphine (4). The compensatory mechanisms apparently are adequate with the result that the autonomic nervous system of a properly stabilized addict is nearly in normal balance (5). When the drug is suddenly removed unbalanced compensatory mechanisms remain and a typical abstinence syndrome appears. While *individual* A.S.I. differences are fairly common on the same dosage level, presumably accountable for on the basis of differences in autonomic stability from patient to patient, in our experience these tend to balance out when *groups* of five or more are studied.

From this viewpoint the T.A.S.-7 is a measure of the total latent autonomic imbalance which existed at the start of the withdrawal. If this be true it

appears that the total autonomic imbalance will exceed a definite maximum value only by the amount of individual variations since the exponential relation found cannot exceed $a = 260$ for any value of dosage. From the form of the function it is evident that doses greater than about 500 mg. will have little additional effect, and experience tends to bear this out.

The values of the constants obtained are of comparatively little importance and depend upon the units and the exact type of scoring system employed. It might appear that the particular scoring system used here would automatically produce the type of relation found, since limits have been placed on several of the components of the syndrome. A careful study of table I however will show that with the exception of the systolic blood pressure rise all signs with limits are those not subject to quantitative measurement. The limit on points for rise in blood pressure is a liberal one, and if exceeded probably indicates an abnormal cardiovascular system. Thus, the saturation value found is not due to a lack of range in the scoring system.

The slope of the curve becomes maximal as zero dosage is approached which suggests that relatively greater autonomic imbalance is obtained from the small, early doses. At the outset the drug is highly effective in small amounts and small increases suffice to offset the antidote effect of beginning tolerance and dependence, but as addiction proceeds the added amounts become less effective. As maximum imbalance is reached the effect of the extra marginal dose becomes practically zero and then the addict must become reconciled to a state of maximum physical dependence with an almost complete loss of the satisfaction originally obtained. Histories obtained from addicts, while quite variable and perhaps uncertain, generally fit well the course deduced above from the shape of the T A S 7 dose curve.

SUMMARY

Data on the abstinence syndrome intensities of 127 morphine addicts stabilized on doses ranging from 40 to 500 mg. per day indicate that a functional relationship exists between the total abstinence syndrome for seven days (T A S 7) and the stabilization dose. From the form of this function it appears that a maximum T A S 7 would be expected at a daily dose of about 500 mg. The theoretical implications and a practical application are discussed.

REFERENCES

1. KOLB I., AND HIMMELSBACH, C. K., Clinical studies of drug addiction. III. A critical review of the withdrawal treatments with method for evaluating abstinence syndromes. *Am J Psychiat* 94 759 1938.
2. HIMMELSBACH, C. K. The morphine abstinence syndrome, its nature and treatment. *Ann Int Med* 16 829, 1912.
3. HIMMELSBACH, C. K., Treatment of the morphine abstinence syndrome with a synthetic cannabis like compound. *South Med J* 37 26 1944.
4. HIMMELSBACH, C. K. With reference to physical dependence. *Federation proceedings* 2 201 1913.
5. HIMMELSBACH, C. K. Clinical studies on drug addiction. Physical dependence with withdrawal and recovery. *Arch Int Med* 69 766 1912.

INHIBITION OF NERVOUS TRANSMISSION IN SYNAPSES AND END PLATES BY THIAMINE

K. UNNA AND E. P. PICK¹

From the Merck Institute for Therapeutic Research, Rahway, New Jersey, and the Mount Sinai Hospital, New York, New York

Received for publication May 24, 1944

In spite of numerous investigations on the pharmacologic action of thiamine in normal and vitamin deficient animals, few data are recorded dealing with the effect of thiamine on the function of the autonomous nerve system. In contrast to striking effects obtained with minute amounts of this vitamin in animals subjected to thiamine deprivation, the early pharmacologic studies of Molitor and Sampson (1) demonstrated that the administration of excessive doses of thiamine produces no significant changes in animals which had been maintained on adequate diets. Since the organism normally disposes rapidly of any excess thiamine by excretion through the kidneys and the intestine, it appears futile to follow the pharmacologic effects of repeated administration of large doses of thiamine in normal animals.

Peripheral nerves are well protected against flooding with thiamine by their myelin sheaths in which according to v. Muralt (2), the thiamine necessary for the functioning of the nerves is mainly localized. Thus, it is hardly to be expected that the normal nerve in its entire length and its endings as far as it is protected by sheaths is susceptible to the administration of large doses of thiamine. However, it would appear that pharmacologic effects of thiamine are more likely to be observed in the less well protected ganglia, in synapses and in analogous receptive apparatus (end plates) of the neuro muscular junction in striated muscles. In this connection it may be recalled that the quantities of thiamine involved in the functioning of peripheral nerves are rather considerable. Following stimulation of motor nerves, v. Muralt (2) found increases in thiamine amounting to 2 micrograms per gram of nerve tissue as compared to a simultaneous increase of 0.1 microgram of acetylcholine. Isolated organs, such as the striated muscle and the small intestine were considered to offer a better opportunity for observing pharmacologic effects of thiamine especially in view of the lack of myelin sheaths in these organs and the impaired metabolism under the conditions of the experiment. The observations reported in the following are intended as a contribution to the mode of action of thiamine on the autonomous nerve system.

METHODS. The experiments on the isolated intestine of guinea pigs and rabbits were carried out in a manner similar to that used in a previous study with sulfonamides (3). Nicotine in the form of nicotine base and thiamine hydrochloride were used in aqueous solutions. The two moieties of the thiamine molecule, 1-methyl-5-hydroxyethylthiazole and 2-methyl-5-ethoxymethyl-6-aminopyrimidine obtained in pure form through the courtesy of Dr. W. H. Engels of the Merck Research Laboratories were tested individually.

¹ Fellow of the Dazian Foundation for Medical Research.

In order to compare the effects of thiamine with other vitamins of the B group, experiments with riboflavin, nicotinamide, pyridoxine, pantothenic acid and para-aminobenzoic acid were included in this study. The addition of thiamine hydrochloride in amounts of 20 mgm. to 100 cc. of Ringer decreased the pH of the solution from 7.55 to 7.0. The addition

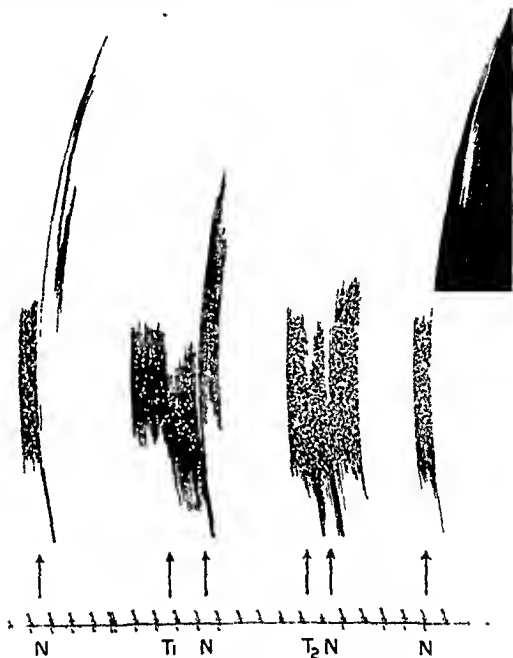


FIG. 1. EFFECT OF THIAMINE ON THE ACTION OF NICOTINE

Isolated intestine of the rabbit. N, nicotine 0.5 mgm. per 100 cc. T₁, thiamine 10 mgm. per 100 cc. T₂, thiamine 15 mgm. per 100 cc.

of the other B vitamins in amounts of 10-50 mgm. per 100 cc. caused changes in the pH of the Ringer solution varying from 7.0 to 8.5. However, control experiments with Ringer solutions adjusted to this range of pH showed that such changes did not alter the reaction of the intestine to nicotine. Other drugs tested in conjunction with thiamine were epinephrine, acetylcholine, lentin and prostigmine.

RESULTS. A. *Experiments on the isolated intestine.* Antagonistic effect of thiamine on nicotine. The addition of thiamine greatly depressed the reaction of the intestine to a subsequent administration of nicotine. On the rabbit's in-

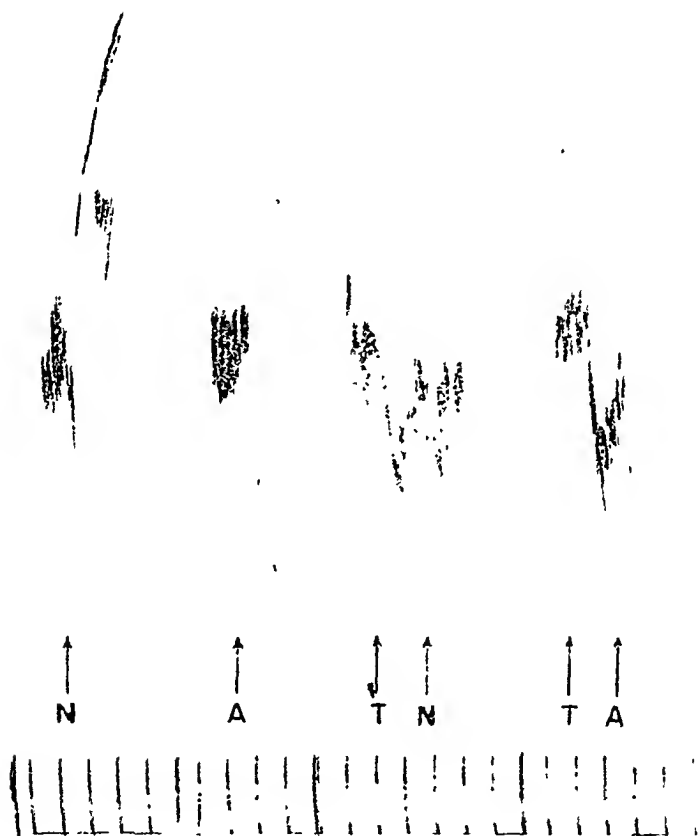


FIG. 2. EFFECT OF THIAMINE ON THE ACTION OF NICOTINE AND ADRENALIN

Isolated intestine of the rabbit. N, nicotine 0.5 mgm. per 100 cc. A, adrenalin 0.1 mgm. per 100 cc. T, thiamine 10 mgm. per 100 cc.

testine concentrations of 2 mgm. to 10 mgm. per 100 cc. were sufficient to decrease the response to nicotine, and 15 mg. of thiamine per 100 cc. regularly prevented the effect of nicotine (fig. 1). In these concentrations, thiamine itself had no significant effect upon the motility of the rabbit's intestine. In the guinea pig's

intestine thiamine was equally effective in preventing the effect of nicotine; however, thiamine alone lessened somewhat the tone of the guinea pigs intestine without altering its rhythmic movements. In most experiments the reaction to nicotine was tested about 2 minutes after thiamine had been added to the Ringer solution. Repeated nicotine tests over a period of 30 minutes following the addition of thiamine showed that the intestine failed to respond to nicotine as

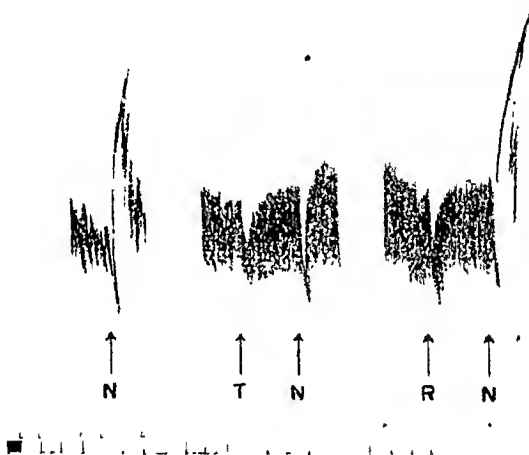


FIG. 3. EFFECT OF THIAMINE AND RIBOFLAVIN ON THE ACTION OF NICOTINE. Isolated intestine of the rabbit. N, nicotine 0.5 mgm. per 100 cc. T, thiamine 10 mgm per 100 cc. R, riboflavin 50 mgm per 100 cc.

long as thiamine was present. Removal of thiamine by changing the bath fluid promptly restored the sensitiveness of the intestine to nicotine.

Coccarboxylase was found as effective as thiamine hydrochloride in antagonizing the effect of nicotine on the isolated intestine.

When the moieties of the thiamine molecule were tested individually, the sulfur free pyrimidine portion failed to influence the nicotine reaction even in concentrations of 100 mg. per 100 cc. On the other hand, with the thiazole moiety in concentration of 15 mgm. to 50 mgm. per 100 cc., a gradually increased inhibition of the nicotine action was obtained. A concentration of 50 mgm. per 100 cc.

completely prevented the effect of nicotine, a result comparable to that obtained with 15 mgm. of thiamine per 100 cc.

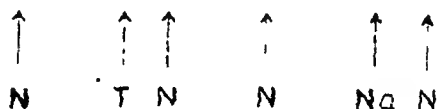


FIG. 4. EFFECT OF THIAMINE AND NICOTINAMIDE ON THE ACTION OF NICOTINE
Isolated intestine of the rabbit. N, nicotine 0.5 mgm. per 100 cc. T, thiamine 10 mgm.
per 100 cc. Na, nicotinamide 50 mgm. per 100 cc.

The addition of small amounts of prostigmine which by themselves did not stimulate intestinal contractions (2.5 to 5 micrograms per 100 cc.) did not exert any influence upon the effect of thiamine on the nicotine reaction.

Effect of thiamine on the action of other drugs. The effect of thiamine on the action of epinephrin, acetylcholine and lentin (carbaminoyle choline) was tested on the isolated rabbit's intestine. In these experiments thiamine was added in concentrations of 10 or 15 mgm. per 100 cc. sufficient to abolish the effect of nico-

tine. No changes in the effectiveness of epinephrin (0.1 mgm per 100 cc) in relaxing the intestine (fig. 2) nor in that of lentin or acetylcholine in stimulating intestinal contractions were observed.

Ineffectiveness of other B vitamins in inhibiting the action of nicotine. None of the B vitamins tested caused any change in the motility of the isolated intestine when given in concentrations of 50 mgm per 100 cc. In contrast to the findings with thiamine, these vitamins failed to influence the reaction to a subsequent administration of nicotine (fig. 3 and 4).

B. Antagonistic effect of thiamine on nicotine on the striated muscle. These experiments were carried out on the isolated abdominal muscle of the frog suspended in frog Ringer. The addition of 10 mgm of thiamine per 100 cc de-

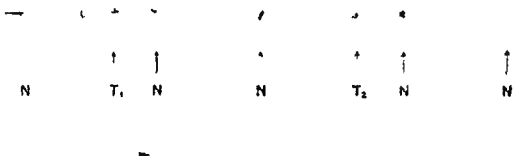


FIG. 5. EFFECT OF THIAMINE ON THE ACTION OF NICOTINE

Isolated abdominal muscle of the frog. N, nicotine 0.5 mgm per 100 cc. T₁, thiamine 10 mgm per 100 cc. T, thiamine 20 mgm per 100 cc.

pressed and 20 mgm abolished the reaction of the muscle to nicotine in doses of 0.5 mgm per 100 cc (fig. 5). These experiments were repeated with Ringer solution to which prostigmine had been added (5 micrograms per 100 cc). However, no influence of prostigmine upon the efficacy of thiamine in inhibiting the action of nicotine was observed. Carboxylase was equally effective as thiamine.

DISCUSSION. The results demonstrate that thiamine in concentrations of 10 to 15 mgm per 100 cc prevents the effect of nicotine on the isolated intestine of rabbits and guinea pigs and on the isolated skeletal muscle of the frog as well. Administration of prostigmine failed to counteract this effect of thiamine. Thiamine in these concentrations temporarily blocks the action of nicotine but causes no permanent damage to the ganglia since the removal of thiamine by changing the nutrient solution restores the response to nicotine. In analogy to previous

experiments with sulfonamides (3), the inhibitory effect of thiamine was found to be confined to the synapses; the action of sympathico and parasympathico mimetic drugs on the nerve endings of the intestine (epinephrin, acetylcholine) remained unchanged. However, in contrast to the findings with sulfonamides, thiamine in inhibiting the nicotine action on the striated muscle, exerts an effect upon the receptive apparatus of the end plates at the myoneural junction. This observation tends to support the opinion of Eccles who, on the basis of his observations on synaptic potentials, concluded that there are no fundamental differences between the transmission of impulses in synapses and that in the myoneural junction (4).

The effect of thiamine upon the action of nicotine is not shared by other B vitamins such as riboflavin, pyridoxine, pantothenic acid, nicotinamide or para aminobenzoic acid. In view of the results obtained with the thiazole and the pyrimidine moieties of thiamine it appears that the effect of thiamine is linked to the thiazole structure of the molecule. It is tempting to speculate that the inhibitory effect both of sulfonamides and of thiamine may in part be based upon the presence of the organic sulfur in these compounds, although thiourea (100 mgm. per 100 cc.) failed to influence the reaction to nicotine. In this connection it may be recalled that thiamine under certain conditions exerts an inhibitory effect upon diamino oxidase (5) and on choline esterase (6).

The action of thiamine upon synapses and myoneural junctions has been demonstrated only in isolated organs following the use of very large doses of the vitamin. It is, however, not impossible that these reactions might be of importance in the physiologic action of thiamine upon metabolic process as involved in the humoral transmission which as yet are not well understood (2).

SUMMARY

1. Thiamine (5-15 mgm. per 100 cc.) inhibits the action of nicotine in the isolated intestine of rabbits and guinea pigs. Cocarboxylase has the same effect as thiamine. The action of drugs stimulating sympathetic or parasympathetic nerve endings is not influenced by thiamine.

2. This effect of thiamine is linked to the thiazole moiety of the thiamine molecule and appears to be analogous to the effect of certain sulfonamides.

3. Thiamine also inhibits the action of nicotine in the striated muscle of frogs.

4. The effect of thiamine upon the nicotine action is not influenced by prostigmine.

5. The processes involved in the inhibition of the nicotine action in synapses and in end plates at the myoneural junction are discussed.

Acknowledgement. Appreciation is expressed to Mrs. Grace R. Peters for her valuable technical assistance.

REFERENCES

1. MOLITOR, H., AND SAMPSON, W. L., *E. Merck's Jahresbericht*, 50, 51, 1936.
2. MURALT V., A., *Nature*, 152, 188, 1943.
3. PICK, E. P., BROOKS, G. W., UNNA, K., *THIS JOURNAL*, 81, 133, 1944.
4. ECCLES, J. C., *J. Physiol.*, 101, 465, 1943.
5. ZELLER, E. A., SCHÄR, B., AND STAEHLIN, S., *Helv. Chem. Acta.*, 22, 837, 1939.
6. GLICK, D., AND ANTROPOL, W., *THIS JOURNAL*, 65, 389, 1939.

SULFAMERAZINE¹ (2-SULFANILAMIDO-4-METHYLPYRIMIDINE)

III THE COMPARATIVE ACTIVITY OF SULFAMERAZINE, SULFADIAZINE AND SULFAPYRIDINE IN THE PRODUCTION OF HEMOLYTIC ANEMIA IN THE MOUSE

ALBERT R. IATVLN AND ARNOLD D. WFLCH

From the Department of Pharmacology, Medical Research Division, Sharp and Dohme, Inc., Glenolden, Pa.

Received for publication May 22, 1944

Hemolytic anemia is occasionally produced when sulfonamides are administered to man (1). In the mouse, however, hemolytic anemia of variable intensity develops within a period of two weeks when sulfanilamide, sulfanilicguanidine, sulfapyridine or sulfathiazole is incorporated in a stock ration (2). The potentiality of each of these sulfonamides for the production of hemolytic anemia was related by Richardson (2) to the concentration of the compound within the erythrocytes. The four sulfonamides were shown to be indistinguishable from one another with respect to the molar erythrocytic concentration required to produce anemia. In this study two pyrimidine derivatives of sulfanilamide, namely sulfamerazine and sulfadiazine, were compared with sulfapyridine as to their ability to produce hemolytic anemia in the mouse. Although Richardson's findings (2) with sulfapyridine have been confirmed, it was observed that sulfadiazine and sulfamerazine produce hemolytic anemia in the mouse only when the sulfonamide concentration within the erythrocytes is raised to a level approximately ten times that required for sulfapyridine. Hemolytic anemia may result from the presence of sulfonamide molecules within the erythrocytes, but all sulfonamides are not equivalent in their anemia-producing potency on the basis of their molar concentration in the blood or in the erythrocytes of the blood. Those sulfanilamide derivatives studied by Richardson (2) produced hemolytic anemia when the molar concentration in the red cells was only approximately one tenth that which we found to be necessary with sulfamerazine or sulfadiazine.

METHODS. Normal healthy mice (18-25 gm. in weight) were kept in groups of 5 on wide meshed screening under conditions of constant temperature and humidity and fed a powdered stock ration.² The sulfonamides were thoroughly incorporated in the diet in various concentrations, each of which was given *ad libitum* to several experimental groups.³ Prior to and at the end of a 2 week period of sulfonamide feeding the hemoglobin content of the blood was determined colorimetrically by the acid hematin method. Blood (0.05 cc.) was drawn from the tail of each mouse and added to $\frac{1}{10}$ hydrochloric acid (12.5 cc.). All photoelectric colorimeter readings were made 24 hours after the preparation of the acid hematin suspensions.³ It was found that 6 hours were required for the readings to attain a maximal value and that thereafter no significant change occurred during a period of 6

¹ Sulfamerazine was originally termed sulfamerazine.

² Purina Dog Chow.

³ For reference the Klett acid hematin standard suspension was employed.

months at refrigerator temperature. At the end of the 2-week period of sulfonamide feeding, blood samples (0.05 cc.) were taken for the determination of the concentration of free sulfonamide according to the method of Bratton and Marshall (3).

RESULTS. Considerable variation in the concentration of sulfonamide was noted in the blood of mice which received the same drug-diet. This was particularly noticeable in mice given the diet containing sulfapyridine, among which the individual differences were approximately three times as marked as in the mice given diets containing sulfadiazine or sulfamerazine. Both before and after sulfonamide feeding, the concentration of hemoglobin in the blood of the mice showed considerable variations from the mean. Because of these variations it was considered unsuitable to attempt to correlate the concentration of sulfonamide in the blood with the change in hemoglobin concentration.

A method found useful for comparing the anemia-producing potency of these sulfonamides involved a statistical evaluation based upon the percentage incidence of anemia at various levels of sulfonamide concentration in the blood. For the purposes of this calculation it was necessary to designate a critical concentration of hemoglobin in the blood, above which mice would be considered normal and below which anemia would be considered to exist. The average hemoglobin concentration in the blood of the various groups of controls (47 mice) was 17.1 gm. per 100 cc. at the beginning of the experiment and 16.9 gm. per 100 cc. at the conclusion of the 2-week period of observation. Each mouse during this period showed to some degree a positive or a negative change in its hemoglobin concentration and the standard deviation of the average of these changes was ± 2.1 gm. per 100 cc. According to these data 1 in 3 animals should show a change greater than ± 2.1 gm. of hemoglobin per 100 cc. of blood, and 1 in 6 animals should show a spontaneous reduction in the hemoglobin content of the blood to an extent greater than 2.1 gm. per 100 cc. Actually only 1 in 11 of the control animals showed a decrease of this magnitude. The diagnosis of anemia was made, therefore, if a decrease in hemoglobin concentration exceeding 2.1 gm. per 100 cc. of blood was found at the end of the 2-week period of sulfonamide feeding. The percentage incidence of such deviations from the hemoglobin level of the control mice was found to be related to the concentration of sulfonamide which occurred in the whole blood or in the erythrocytes. In table 1 are presented the data and the calculations from which the curves presented in figure 1 were derived. It will be seen that as the concentration of sulfonamides in the blood increased a progressive increase in the incidence of anemia was encountered. With a concentration of sulfapyridine of 8 mgm. or more per 100 cc. of blood *all* mice showed a hemoglobin decrease greater than 2.1 grams per 100 cc. of blood. With sulfamerazine and sulfadiazine, however, a sulfonamide concentration of approximately 60 mgm. per 100 cc. was required to produce a 100 per cent incidence of anemia. Since comparisons of drugs which produce a given pharmacological effect can be made most accurately at dosage levels affecting approximately 50 per cent of the animals, the "Anemia-Producing Concentration₅₀" (A.P.C.₅₀) was calculated by Behren's method (4). In figure 1 is shown the A.P.C.₅₀ for each of the three sulfonamides. The A.P.C.₅₀ of sulfa-

TABLE I

Incidence of anemia in mice receiving sulfapyridine, sulfamerazine and sulfadiazine in the diet

AVERAGE BLOOD CONCN	RANGE OF BLOOD CONCN	NO OF ANIMALS		BERREN'S METHOD		PER CENT OF ANIMALS ANEMIC
		Normal	Anemic	Normal	Anemic	
Sulfapyridine						
<i>mgm per cent</i>	<i>mgm per cent</i>					
0.1	0.0-0.2	2	0	55	0	0
0.5	0.3-0.7	10	1	53	1	2
1.0	0.8-1.2	9	4	43	5	10
1.5	1.3-1.7	6	4	34	9	21
2.0	1.8-2.2	0	5	28	14	33
2.5	2.3-2.7	5	2	19	16	46
3.0	2.8-3.2	1	2	14	18	56
3.5	3.3-3.7	2	2	13	20	61
4.0	3.8-4.2	2	2	11	22	67
4.5	4.3-4.7	4	3	9	25	74
5.0	4.8-5.2	1	1	5	20	84
5.5	5.3-5.7	2	1	4	27	87
6.0	5.8-6.4	1	2	2	29	94
7.0	6.5-7.4	1	0	1	29	97
8.0	7.5-8.4	0	3	0	32	100
	8.5-31.0	0	25	0	57	100
Sulfamerazine						
10.0	8-12	2	0	23	0	0
15.0	13-17	3	2	26	2	7
20.0	18-22	6	1	23	3	12
25.0	23-27	4	4	17	7	29
30.0	28-32	4	4	13	11	46
35.0	33-37	5	3	9	14	61
40.0	38-42	2	2	4	16	80
45.0	43-47	0	2	2	18	90
50.0	48-52	1	0	2	18	90
60.0	58-62	1	1	1	19	95
65.0	63-67	0	1	0	20	100
	68-115	0	5	0	25	100
Sulfadiazine						
10.0	8-12	2	0	23	0	0
15.0	13-17	5	0	21	0	0
20.0	18-22	3	0	16	0	0
25.0	28-27	3	5	13	5	28
30.0	28-32	2	3	10	8	44
35.0	33-37	3	1	8	9	53
40.0	38-42	2	1	5	10	67
45.0	43-47	0	1	3	11	79
50.0	48-52	3	3	3	14	82
60.0	58-62	0	2	0	16	100
	63-115	0	2	0	18	100

pyridine (2.8 mgm. per 100 cc. of blood) indicates the great potentiality of this drug for the production of anemia in mice. However, with sulfamerazine or sulfadiazine anemia was not produced until very high concentrations in the blood were attained; the A.P.C.₅₀ of sulfamerazine was 31.0 mgm. per 100 cc., while that of sulfadiazine was 33.0 mgm. per 100 cc. of blood.

Although microscopic examination of the blood was not done routinely, it was noted that the number of reticulocytes varied inversely with the hemoglobin concentration—a finding which strongly suggests that the anemia was of the hemolytic type. Large numbers of erythrocytes containing Heinz bodies were also observed. These changes in the erythrocytes were of the same general character whether produced by sulfamerazine, sulfadiazine or sulfapyridine,

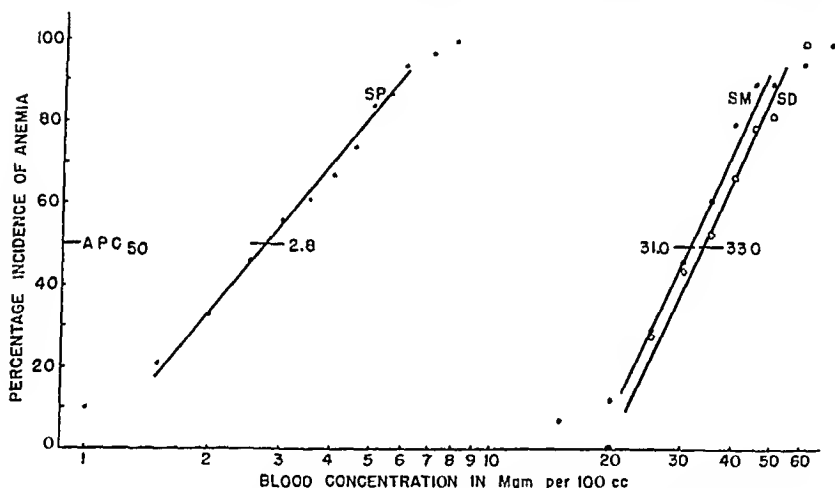


FIG. 1. THE INCIDENCE OF ANEMIA IN MICE AFTER RECEIVING SULFAPYRIDINE (SP), SULFAMERAZINE (SM), OR SULFADIAZINE (SD) IN THE DIET FOR A PERIOD OF TWO WEEKS

and were similar to those reported by Richardson (2, 5) with sulfanilamide, sulfapyridine, sulfathiazole, sulfanilylguanidine and diaminodiphenylsulfone.

The micromolar concentrations of the sulfonamides within the erythrocytes, calculated from the hematocrit and the whole blood and plasma sulfonamide concentrations,⁴ showed that the relation between the three compounds studied closely resembled that found in whole blood. Preliminary observations indicate that in mice in which a sulfonamide concentration sufficient to cause a 50 per cent incidence of anemia is present in the blood, the concentration in the erythrocytes is higher in the anemic mice than in the non-anemic members of the group. In other words, with the occurrence of anemia the ratio of erythrocytic concentra-

⁴ $C_E = C_B - [C_P(1 - H)]/H$, where C_E = concentration (mgm. of sulfonamide per 100 cc.) in erythrocytes, C_B = concentration in whole blood, C_P = concentration in plasma, and H = hematocrit expressed as a decimal fraction of 1.

tion to plasmic concentration appears to increase. Since the anemic mice showed a high degree of reticulocytosis it was thought that the higher drug concentration in the red cells conceivably might be accounted for by a higher content in the immature reticulum containing cells. The data available, however, show that the uppermost portion of a column of centrifuged erythrocytes in which reticulocytes were concentrated, naturally contained less sulfonamide per unit volume than the lowest portion of the column which was relatively free of reticulocytes. The difference in sulfonamide concentration between the two layers of centrifuged erythrocytes did not result from a difference in the packing of the red cells; no significant difference was found when the same experiment was repeated with the blood of normal mice two hours after the oral administration of a single dose of sulfonamide. Further study will be required to determine whether the increased ratio of erythrocytic to plasmic concentration of sulfonamide in anemic mice is of primary or of secondary significance.

Discussion. At present it is not possible to offer an explanation for the fact that the production of anemia requires a very much higher concentration of sulfadiazine or sulfamerazine than of sulfapyridine and the other sulfonamides. It appears that the two pyrimidine derivatives behave differently from those sulfonamides investigated by Richardson (2), namely sulfanilamide, sulfathiazole, sulfapyridine, and sulfamylguanidine. Since Richardson found these four compounds to be equally productive of hemolytic anemia in the mouse when the molar concentration in the erythrocytes was considered, it follows that in this respect each is about ten times as toxic as sulfamerazine or sulfadiazine.

It is interesting to consider whether these observations made in mice bear any relation to observations made in human subjects. In their study of the toxic reactions produced by sulfonamides in man, Dowling and Lepper (1) observed 7 cases of acute hemolytic anemia among a total of 508 patients treated with sulfapyridine, while among 660 cases treated with sulfadiazine there was only 1 case of acute hemolytic anemia. Certainly these series of cases are not sufficiently large to permit accurate statements concerning the comparative incidence of hemolytic anemia in man following the use of these two drugs. On a percent age basis, the data of Dowling and Lepper (1) show that the incidence of hemolytic anemia in their sulfapyridine treated cases (1.4 per cent) was about nine times that in their sulfadiazine treated cases (0.15 per cent). It will be recalled that in mice sulfapyridine is productive of anemia when the concentration in the erythrocyte is only about one tenth that required with sulfadiazine or sulfamerazine.

Although we have not studied the anemia producing effects of sulfathiazole in the mouse, Richardson (2) found that on a basis of the micromolar concentration within the erythrocyte, this drug was comparable to sulfapyridine in its ability to produce hemolytic anemia. Dowling and Lepper (1) found only 1 case of acute hemolytic anemia among 321 patients given sulfathiazole. On a percent age basis (0.31 per cent) this incidence is about twice that of sulfadiazine, however, the average concentration of sulfathiazole in the blood in the human cases was significantly lower than that of sulfadiazine and the number of cases is much too small to suggest that the difference has significance.

Obviously, a very much larger number of cases will be required to prove conclusively that in man, as in the mouse, sulfadiazine is markedly less productive of hemolytic anemia than are sulfathiazole and sulfapyridine. The present extensive clinical usage of sulfamerazine should soon permit a conclusion concerning its productivity of hemolytic anemia in man. Data from its use, in comparison with data obtained using other sulfonamides, will indicate further whether the measurement in the mouse of the potentiality of a sulfonamide for the production of hemolytic anemia is of importance in the experimental evaluation of new members of this group of chemotherapeutic agents.

SUMMARY

The hemolytic anemia-producing properties of sulfapyridine, sulfamerazine and sulfadiazine have been evaluated in mice by determining the percentage incidence of anemia produced by various concentrations of sulfonamide in the blood. The concentration of sulfonamide in the blood necessary to produce a 50 per cent incidence of anemia was found to be: with sulfapyridine, 2.8 mgm., with sulfadiazine, 33.0 mgm., and with sulfamerazine, 31.0 mgm. per 100 cc. Although a high micromolar concentration of sulfonamide within the erythrocytes was required to produce anemia with sulfadiazine and sulfamerazine, sulfapyridine produced the same incidence of anemia with an erythrocytic concentration only about one-tenth as great. The possible clinical significance of these findings is discussed.

BIBLIOGRAPHY

1. DOWLING AND LEPPER, *J. A. M. A.*, **121**, 1190, 1913.
2. RICHARDSON, *THIS JOURNAL*, **72**, 99, 1941.
3. BRATTON AND MARSHALL, *J. Biol. Chem.*, **123**, 537, 1939.
4. BEHRENS, *Arch. exptl. Path. Pharmac.*, **140**, 237, 1929.
5. RICHARDSON, *THIS JOURNAL*, **67**, 429, 1939.

THE PHARMACOLOGICAL BASIS FOR THE RATIONAL USE OF ATABRINE IN THE TREATMENT OF MALARIA¹

JAMES A SHANNON, DAVID P EARLE, JR., BERNARD B BRODIE, JOHN V TAGGART, ROBERT W BERLINER AND THE RESIDENT STAFF OF
THE RESEARCH SERVICE²

From The Research Service, Third (New York University) Medical Division, Goldwater Memorial Hospital, and The Department of Medicine, New York University College of Medicine

Received for publication January 7, 1944

Present usage of atabrine in the suppression and treatment of malaria is largely empirical. Until recently (1), regimes of therapy appeared to be constructed so as to obtain a therapeutic effect roughly equivalent to that of quinine and at the same time to minimize the hazard of toxic reactions (2). Such an approach to the general problem of atabrine therapy is a striking contrast to the more quantitative one which has facilitated the development of sound antibacterial therapy with the sulfonamides. The availability of relatively simple methods for the estimation of atabrine concentration in biological fluids (3, 4) now permits the latter type of approach to the problems of atabrine therapy.

The observations transmitted in this report are derived from studies which examine certain aspects of the fate of atabrine in the body under several general circumstances. The fundamental premise, underlying these and other similar studies, is that the antimalarial activity of atabrine may be related to its concentration in the plasma, or, perhaps more precisely, to the concentration of unbound drug in plasma water. Some general support of this thesis will be found in the data of this report. More direct evidence will be presented in other communications (5).

The report is divided into three sections. The first is concerned with the physiological disposition of atabrine in the body. It details observations made on the dog and on man which outline that part of the general pharmacology of

¹ The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and New York University.

The work has been described in full in a memorandum prepared for the Committee on Medical Research and submitted July 15 1943. The memorandum is entitled, "On the Use of Atabrine in the Treatment of Malaria."

² The resident staff who rendered invaluable assistance at various times during the study consisted of Roger L. Greif, Lieutenant (jg) MC, USNR, Hugh A. Miller, Lieutenant Commander MC V(G), USNR, Jackson Norwood, Lieutenant (sg) MC, USNR, William J. Welch, 1st Lieutenant MC AUS, Bowman Wise, Charles G. Zubrod, 1st Lieutenant, MC, AUS.

atabrine which is of importance in conditioning its specific therapeutic effect. The second section consists of an examination of the use of atabrine as a suppressive antimalarial agent. The third section is concerned with the use of atabrine in the treatment of clinical malaria. These investigations are not designed to obtain a clinical evaluation of the effectiveness of the therapeutic regimes examined. Rather, the data as a whole should serve as a background of information for use in the construction of rational regimes of therapy. These may then be examined in larger scale studies, and finally tested for clinical effectiveness in naturally occurring malaria.

SECTION I. THE PHYSIOLOGICAL DISPOSITION OF ATABRINE IN THE BODY. Outstanding characteristics of the physiological disposition of atabrine are a rapid and essentially complete absorption from the gastro-intestinal tract, a low rate of excretion, a low rate of degradation and a tendency to extensive localization in many organs of the body. The properties of the drug which relate to its absorption, degradation and excretion have been sketched in by simple experiments or by the use of information drawn from the recent investigations of others. The distribution of atabrine in the body has been re-examined since previously available data do not include observations which relate the concentration of the drug in the various tissues to a reference fluid such as plasma or plasma water (c.f. 6). The data presented are adequate to characterize the salient features of the physiological disposition of atabrine but do not constitute an exhaustive inquiry into the details of the several processes involved.

Experimental. Chemical method: The chemical method used in these observations is a double extraction procedure recently described. The initial ethylene dichloride extracts were, in all cases, washed with alkali so that the values given in the tables refer to atabrine as such and do not include any of its fluorescent degradation products (4, 7).

Preparation of biological samples: Plasma: Special precautions must be taken in the preparation of the various partitions of the blood for analysis due to the unequal distribution of atabrine in this tissue (table 2). The technique used in the preparation of the plasma samples is as follows: Blood is drawn using adequate amounts of oxalate as an anticoagulant. It is immediately centrifuged at 1500 r.p.m. for fifteen minutes, the upper portion of the plasma is removed and recentrifuged for an additional hour at 1500 r.p.m. This procedure is designed to minimize the contamination of the plasma sample by atabrine derived from the leucocyte.

Leucocytes and Erythrocytes: Several samples of blood are centrifuged in 25 ml. tubes at 1500 r.p.m. A sample of leucocytes is obtained by pooling the buffy coats separated from the series of blood samples and recentrifuging the pooled cells in a clean tube at 1500 r.p.m. until a sharp separation is effected between the leucocytes and other layers. The supernatant plasma is removed by aspiration and aliquots of the leucocytes are then weighed out for analysis. A sample of erythrocytes is obtained by taking a centrifuged blood sample from which the plasma and leucocytes have been removed and pipetting an aliquot from the bottom of the packed erythrocytes.

Tissues: Small pieces of tissue are removed from freshly sacrificed animals. The samples are weighed and prepared for analysis by homogenizing in a motor driven glass device which produces fracture of most cells (4).

Urine and Fees: Appropriate amounts of each of these are taken from 24 hour collections and the atabrine shaken out directly into the ethylene dichloride as in the plasma de-

termination The 24 hour collection of feces is first converted into a fine suspension in dilute hydrochloric acid by vigorous shaking

Measurement of plasma binding An indirect approach to the measurement of plasma binding is necessary since atabrine is extensively bound on the membranes commonly used for ultrafiltration and dialysis The error due to this phenomenon is particularly large at the concentrations of unbound atabrine which are commonly encountered in the plasma water during most regimes of therapy

The indirect method consists of the determination of the concentration of atabrine in a buffered Ringer's solution which produces the same equilibrium concentration of atabrine in a sample of erythrocytes as obtains when the erythrocytes are equilibrated with a given concentration of atabrine in whole plasma The equilibrations are performed at 22° in order to minimize hemolysis A summary of an experiment on dog plasma is given in table 1 Such a procedure may not yield a precise expression of the situation which obtains *in vivo* due to the temperature at which the equilibration is performed However, the values obtained may be accepted as close approximation of these relationships

TABLE 1

Plasma binding of atabrine

The extent to which atabrine is bound to the non diffusible constituents of plasma was approximated as in the following experiment

Atabrine was added in various amounts to measured aliquots of a sample of dog plasma and buffered Ringers solution Aliquots of a single sample of dog erythrocytes were then added to each of the above fluids The cell suspensions were equilibrated at 22°C for one hour with occasional agitation, they were then centrifuged and the separated cells and supernatants analysed for atabrine in the usual fashion

SAMPLE STUDIED	ATABRINE ADDED	ATABRINE CONCENTRATION		RATIO CELLS SUPERNATANT	PLASMA BINDING
		Supernatant	Erythrocytes		
	<i>micrograms</i>	<i>micrograms per liter</i>	<i>micrograms per liter</i>		<i>per cent of total concentration</i>
Plasma	1 0	21	50	2 4	70
Plasma	1 5	64	143	2 2	72
Plasma	3 0	122	280	2 1	71
Plasma	7 5	336	721	2 2	73
Plasma	15 0	615	1472	2 4	72
Ringers	1 5	28	230	8 2	
Ringers	7 5	100	701	7 9	

Experimental results The distribution of atabrine in whole blood Data on the distribution of atabrine in the blood of two patients are summarized in table 2 These blood samples were obtained after the administration of 0.1 gram of atabrine dihydrochloride three times daily for many days The results presented are typical of what has been found on other samples of blood from both canine and human subjects The concentration of the atabrine in erythrocytes is usually one to two times that of plasma, whereas, the concentration in leucocytes is in excess of two hundred times the plasma concentration It is impossible to obtain a pure sample of either general cell type Consequently, the atabrine

concentrations given for leucocytes are lower than actually occur and the concentrations given for erythrocytes higher.

Such a distribution in the blood is rather unusual and has important implications. It is in consequence of this distribution that information on the atabrine concentration of whole blood in any situation is of little use to the pharmacologist or the clinician. It has been noted, when wide variations in the leucocyte count are encountered during a course of atabrine therapy, that the whole blood atabrine concentration is more apt to reflect the change in the leucocyte count than the underlying plasma atabrine concentration. On the other hand, considerable variations have been encountered in the relationship between the concentration of atabrine in the whole blood and in plasma which are not completely accounted for on the basis of variations in the leucocyte count alone or the absolute concentration in the partitions of blood examined. These findings suggest that other

TABLE 2

Distribution of atabrine in human blood

A summary of the distribution of atabrine in the blood of two subjects receiving 0.1 gm. of the dihydrochloride three times daily. Note should be taken of the fact that the cell samples are mutually contaminated. The simultaneously observed concentration of atabrine in cerebrospinal fluid is also given because of the relationship it may be expected to bear to the concentration of atabrine in the plasma water.

	ATABRINE CONCENTRATION (MICROG./LITER)	
	Subject Ma	Subject Go
Plasma	90	89
Plasma water	14.6	8.9
Cerebrospinal fluid	4.3	5.4
Erythrocytes	149	117
Leucocytes	9,500	18,400
Whole blood	291	551
Plasma binding (per cent total)	83	90

factors, which may relate to the time of exposure to atabrine or to the activity of the specific tissue involved (leucocytes in this instance) are concerned in determining the extent to which atabrine is localized at any given concentration of unbound atabrine in plasma water.

An appreciation of the nature of the distribution of atabrine in the blood is also essential if valid estimations of the plasma atabrine concentration are to be obtained. The localization of atabrine in the leucocytes appears to be reversible, at least in part. It has been observed that when shed blood is let stand at room temperature, there occurs a progressive release of atabrine from the leucocytes. This is reflected in a progressive increase in the concentration of atabrine in the plasma. It follows from this that a value, which is truly representative of the *in vivo* plasma atabrine concentration, can only be arrived at when the plasma is separated in a manner which avoids contamination of the plasma sample by leucocytes or leucocyte fragments and which does not permit

the diffusion outwards of a significant amount of the atabrine contained in the leucocytes

A second important characteristic of the distribution of atabrine in the blood is the degree to which it is bound to the non diffusible constituents of plasma, presumably plasma albumin. The extent of this binding in the case of human plasma is in the order of 80 to 90 per cent of the total plasma concentration (table 2). This does not vary with the absolute concentration of atabrine in the usual range of levels that are encountered during therapy.

An appreciation of the state of atabrine in the plasma is important in all theoretical considerations relating to the specific or general pharmacology of atabrine. The concentration of unbound atabrine in plasma water is presumably equal to that of extracellular fluid, and is the equilibrium concentration of the body as a whole, or, as viewed in another light, it is a reflection of the extent to which atabrine is reversibly localized in a series of organs. Either of these general views is justified by the fact that all exchanges of atabrine, whether from blood to organs or from organ to organ, must proceed through this common matrix. Furthermore, the concentration of unbound atabrine in plasma water is the concentration with which parasites or the parasitized erythrocytes of the blood are in equilibrium. And lastly, it represents the concentration of atabrine which is presented to the various renal mechanisms and which, with these, determines the rate of the renal excretion of the drug.

It is not feasible, because of practical considerations, to determine the concentration of unbound atabrine in plasma water as a routine measure, nor is this necessary for most purposes. It is to be anticipated that the bound fraction will be proportional to the plasma albumin concentration and will remain reasonably constant so long as the plasma albumin concentration remains normal. Consequently, unless evidence to the contrary is obtained in further investigations of this aspect of the problem studies on the general pharmacology of atabrine or on the use of atabrine as an antimalarial agent may relate its action to the concentration in whole plasma.

Physiological Disposition. The distribution of atabrine in the body has been examined in a series of experiments performed on dogs which are similar to the ones summarized in table 3.

Atabrine (10 mg per kg) was administered to Dog A by a slow intravenous injection. The atabrine concentration of the plasma was determined at ten minutes and one and a half, and four hours later. The animal was sacrificed shortly thereafter. The apparent volume of distribution of atabrine in Dog A, at four hours, is many times the body weight of the animal and this circumstance is reflected in the high tissue/plasma concentration ratios determined at that time. The degree of the localization of the atabrine in the tissues would be further emphasized by including in the calculation of the ratios the extent to which atabrine is bound on plasma protein.

It may be assumed that diffusion equilibrium, in the absence of specific localization, is achieved in such experiments well within four hours. Consequently, the low initial plasma atabrine concentration, together with the subsequent fall

to an even lower level, is a reflection of rapid, progressive, and extensive localization in various organs. It is also apparent that a dynamic equilibrium has not been achieved in Dog A at four hours. This is indicated by the continued rapid fall of the plasma atabrine concentration which is observed in similar experiments after four hours, and, the extraordinarily high tissue/plasma concentration ratios which are invariably reached when atabrine is administered in repeated daily doses until equilibrium is established (Dog B, table 3). The equilibrium in the latter type of experiment is between processes of absorption, localization, degradation and excretion and the concentration of atabrine in the plasma, or, as noted above, the concentration of unbound atabrine in plasma water.

The distribution of atabrine in the human subject was studied, using the expedient of measuring its apparent volume of distribution following the intra-

TABLE 3
Distribution of atabrine in the dog

Two experiments which examine the distribution of atabrine in some selected tissues of the dog. The studies on Dog A were made four hours after a single injection of atabrine, on Dog B after the daily administration of atabrine for a period of fourteen days.

	DOG A. WT. 15.5 KG.	DOG B. WT. 10.0 KG.
	:00 10 mg/kg. atabrine intravenously :10 Plasma atabrine 0.310 mg./kg. 1:40 Plasma atabrine 0.090 mg./kg. 4:10 Plasma atabrine 0.041 mg./kg.	20 mg. per kg. daily for 14 days prior to experiment. Last dose 14 hours before sacrificing
	Concentration of atabrine (milligrams per kg.)	
Plasma.....	0.041	0.061
Muscle.....	6.80	55.0
Lung.....	22.8	310.0
Spleen.....	161.0	571.0
Liver.....	70.0	1306.0

venous administration of a known amount. A typical experiment showed a plasma atabrine concentration of 30.8 micrograms per liter four hours after the intravenous administration of 200 milligrams of atabrine dihydrochloride to a man weighing 70 kg. The apparent volume of distribution at this time had a value of 6,500 liters or approximately ninety times the body weight. These data indicate that, as in the dog, there is extensive localization of atabrine in the tissues of the human subject. This finding is in keeping with the phenomenon of accumulation which is so manifest in the human subject when receiving small repeated doses of atabrine (Sections II and III).

Excretion is of little importance in determining the plasma atabrine concentrations in these experiments. The normal dog will usually excrete in 24 hours no more than one percent of the amount of atabrine administered in a single dose of the size given in Dog A and approximately 5 percent of the daily dose required to maintain a plasma concentration at the general level observed on

Dog B (see also 6) A similar situation obtains in man, table 4 (see also 8) However, it is of some interest to note that these low excretion rates are largely attributable to the very low concentrations of unbound atabrine in the plasma water rather than to renal factors per se

Degradation is similarly of little importance in determining the relationships observed in experiments of short duration similar to the one on Dog A However, degradation is important in experiments which involve the serial adminis-

TABLE 4

Absorption and excretion of atabrine in man

These observations are examples of the relationships which obtain during the administration of therapeutic doses of atabrine dihydrochloride Plasma atabrine concentrations are included to indicate the extent to which a stable balance has been struck between the absorption, degradation and excretion of atabrine in each subject during the collection of the data Each patient had received 0.1 gm atabrine dihydrochloride three times daily for some days previously Variations observed in the fecal excretion of atabrine are to be expected due to the error involved in sampling

DAY OF OBSERVATION	DAILY ATABRINE	PLASMA CONCENTRATION	RENAL EXCRETION	FECAL EXCRETION
Patient Wa				
	mgm	micrograms per liter	mgm per day	mgm per day
1	300	64		
2	300	103	10.8	18
3	300	87	13.7	20
4	300	92	11.5	15
5	300	96	10.4	12
6	300		6.0	20
7			5.1	10
8				23
Patient St				
1	300	36		
2	300	23		
3	300	32	4.8	30
4	300	48	5.5	7
5		37	7.3	36
6		17	3.3	10
7			8.0	6
8			2.2	11

tration of atabrine over a number of days The plasma atabrine concentration, in these situations, increases abruptly with each dose However, the low rate of degradation, makes possible the progressive accumulation of atabrine in the body which in turn is reflected in a progressive increase in the plasma concentration of atabrine which is maintained between doses The rate of degradation appears to be a function of the plasma concentration since ultimately the basic level of the plasma atabrine concentrations stabilizes The level at which each

individual stabilizes is related to the atabrine dosage and to the rate at which the individual degrades the drug; the latter being different for different individuals. This circumstance follows from the fact that in the normal individual absorption is essentially complete and excretion is sufficiently low to be unimportant. Consequently, when stabilization has been attained the amount of drug degraded each day is approximately equal to the daily dose.

There is considerable variation in these relationships from dog to dog and even greater variation when man is compared to the dog. However, observations on the absorption and excretion of atabrine in human subjects, some examples of which are summarized in table 4, and, the data detailed in Sections II and III, indicate that differences between the dog and man are quantitative rather than qualitative.

Discussion. These studies serve to outline the important factors which are concerned with the physiological disposition of atabrine. The distribution of atabrine in the blood is such that observations on its specific antimalarial action should be related to the concentration of the atabrine in the plasma and perhaps, indirectly from this, to the concentration of the atabrine in plasma water. It is also clear that the plasma atabrine concentration achieved after single or repeated doses is dominated by the tendency of the organs of the body to localize the material within them, and by the slow rate at which the drug is degraded. These characteristics are reflected in the low plasma atabrine concentration which is reached after a single dose of atabrine as well as in the low rate of renal excretion. They also, together with the low excretion rate, are reflected in the slow rate of fall of the plasma atabrine concentration after a single dose and the progressive accumulation of the drug in the body when repeated doses are given over a period of days or weeks.

An appreciation of the above factors is of practical importance since it is the operation of these which regulate the plasma atabrine concentration on any regime of therapy and which, consequently, are reflected in the antimalarial effectiveness of any given regime of atabrine therapy.

SECTION II. AN EVALUATION OF ATABRINE SUPPRESSIVE THERAPY THROUGH A STUDY OF THE PLASMA ATABRINE CONCENTRATIONS ACHIEVED ON SEVERAL DOSAGE SCHEDULES. A study of the plasma atabrine concentrations achieved in individuals on a variety of dosage regimes was undertaken in order to determine the importance of the phenomena described in Section I to the problem of constructing rational regimes of suppressive therapy. Preliminary observations were made on a small series of hospital patients. These received 0.2 gm. of atabrine dihydrochloride twice weekly for periods varying from three to eight weeks (9). It appeared that, on such a dosage schedule, there is a progressive accumulation of atabrine in the body and that this accumulation is reflected in the progressive increase in plasma atabrine concentration. The equilibrium between the oral administration of atabrine and plasma atabrine concentration was not reached, in these observations, until some weeks after beginning atabrine. Furthermore, extensive variation was encountered in the plasma atabrine concentrations of the individuals of the group. These results were of

sufficient practical importance that the study has been extended to include other regimes of therapy on small groups of normal young adults

Experimental Chemical method The plasma samples were prepared as described in Section I. The concentration of atabrine was estimated by the double extraction procedure but did not include an alkaline wash of the ethylene dichloride extract (4). A small quantity of fluorescent degradation of atabrine is, therefore, included in each estimation. However, the error involved is only a few per cent and may be neglected in considering the data of this section. The low plasma atabrine concentrations frequently encountered during suppressive therapy make it necessary to observe every precaution in the use of the chemical method, i.e. in the preparation of reagents, in the manipulations during the estimation and in the maintenance of the fluorimeter at high sensitivity.

Clinical material Volunteers from the first and second year classes of the New York University College of Medicine were used. They may be considered to be reasonably healthy young adults leading a rather sedentary life. Three of the subjects were dropped from the study because of moderately severe gastrointestinal reactions, two of the subjects because of unusually high plasma atabrine concentrations. The latter two subjects had no adverse reactions. However, in the absence of information at the time that concentrations in excess of 50 micrograms per liter are safe over extended periods of time, the drug was withdrawn in these instances. These five subjects are not included in the means of the summary figures.

Regimes of therapy The atabrine was taken on the days indicated below at 1 P.M., immediately after lunch. The dosage schedules and the times of blood sampling were as follows:

1. 400 mg of atabrine dihydrochloride weekly. This was administered in two, 200 mg doses taken on Tuesday and Thursday. Plasma atabrine concentration was estimated in blood samples obtained at 1 and 5 P.M. on each of the days that atabrine was administered.

2. 400 mg of atabrine dihydrochloride weekly. This was administered in four, 100 mg doses taken on Tuesday, Wednesday, Thursday and Friday. Plasma atabrine concentration was estimated in blood samples obtained at 1 P.M. on Tuesday and Thursday, and at 5 P.M. on Friday.

3. 600 mg of atabrine dihydrochloride weekly. This was administered in three, 200 mg doses taken on Wednesday, Thursday and Friday. Plasma atabrine concentration was estimated in blood samples obtained at 1 and 5 P.M. on Wednesdays and Fridays.

4. 600 mg of atabrine dihydrochloride weekly. This was administered in six, 100 mg doses taken on Monday, Tuesday, Wednesday, Thursday, Friday and Saturday. Plasma atabrine concentration was estimated in blood samples obtained at 1 P.M. on Monday and Wednesday and at 5 P.M. on Friday.

5. 400 mg atabrine dihydrochloride weekly. This was administered in 50 mg doses taken on six consecutive weekdays and one 100 mg dose on Sunday. Plasma atabrine concentration was estimated in blood samples taken at 1 P.M. on Tuesday and Friday.

6. 400 mg atabrine dihydrochloride weekly. This was administered as in the previous schedule (number 5) but was preceded by the administration of 1 gm of atabrine dihydrochloride in five, 200 mg doses taken on consecutive days. Plasma atabrine concentration was estimated in this group of patients just preceding the first 50 mg dose of atabrine dihydrochloride and at 1 P.M. on the Monday and Thursday of each week thereafter.

7. 600 mg of atabrine dihydrochloride weekly. This was administered in three, 200 mg doses taken on Monday, Wednesday and Friday. Plasma atabrine concentration was estimated in blood samples obtained at 1 P.M. on Monday and Friday and at 5 P.M. on Friday.

The subjects on regimes 1 to 4 were observed as above for a period of seven weeks, except that observations were not made during the fifth week. A limited number were continued for an additional six weeks. Single blood samples were obtained on some of the latter subjects at the mid period of this interval and the usual observations made during the final week of therapy, i.e., the thirteenth week. The subjects on schedules 5 to 7 were

followed closely for an initial three-week period. A portion of these also continued to take the drug during the following six weeks. Observations were made on some of the latter group at the mid-period and the usual blood samples were obtained during the ninth week of therapy.

The schedules of blood sampling, in relation to the schedule of drug administration in regimes 1 to 4 and 7 were designed to evaluate the minimal and maximal plasma concentrations of each individual during each week. Maximal plasma atabrine concentrations were not determined in the individuals on regimes 5 and 6 because of the small size of the doses. These produce little absolute change in the plasma atabrine concentration with each dose and the change in concentration must be assayed by observations extending over a period of days or weeks. It has been assumed, for the purposes of this study, that a maximal plasma atabrine concentration is reached approximately four hours after the administration of one or two tablets. More detailed observations indicate that there is considerable variation in the time required for this to occur. However, the concentration at this time is usually a fair approximation of the maximal level reached. The weekly minimal plasma atabrine concentration has been taken as that which obtains at the end of the longest drug-free interval.

Experimental results. Figures 1 and 2 are general summaries of the regimes studied. The large dots represent the weekly mean minimal plasma atabrine concentrations, the open circles the weekly mean "maximal" concentrations. The trends are indicated by the solid and broken lines which connect the dots and the circles respectively. The range of variation in the minimal values is indicated by the small dots above and below each mean minimal concentration. The intermediate values which were determined add little to the data and are omitted in the summary figures.

Further details are given in figure 3 and in table 5. The minimal weekly values for each subject on regime 4 are summarized in figure 3. These data illustrate the progressive increase of plasma atabrine concentration in the individuals during the initial weeks of therapy. Table 5 is a summary of the detailed data obtained from the group on regime 2. These detailed data are typical of those obtained with the other groups.

A consideration of the data in relation to that presented in the previous section permits certain general statements.

1. Atabrine progressively accumulates in the body when 0.4 or 0.6 gm. of the dihydrochloride are administered weekly. The accumulation is reflected in a progressive increase of the plasma atabrine concentration which continues over a number of weeks. Eventually a stable concentration is achieved and maintained. The equilibrium concentrations on the 400 mg. regimes are definitely lower than on the 600 mg. regimes. However, the groups are too small to establish the equilibrium concentrations which characterize each regime of therapy.

More detailed studies were made on the plasma atabrine concentrations which follow the individual doses. These indicate that the rate of fall in the plasma level subsequent to attaining a maximal value is rather low. This finding is of consequence in the evaluation of the antimalarial protection conferred by a regime of therapy during the initial weeks since the minimal plasma concentrations are not a true indication of the antimalarial protection conferred during this interval. The minimal plasma atabrine concentration increases progressively as sup-

pressive therapy is continued, and, the increase in plasma concentration becomes proportionately less with the rise in the predose level. The amount of anti-malarial protection conferred by a regime of suppressive therapy is, therefore,

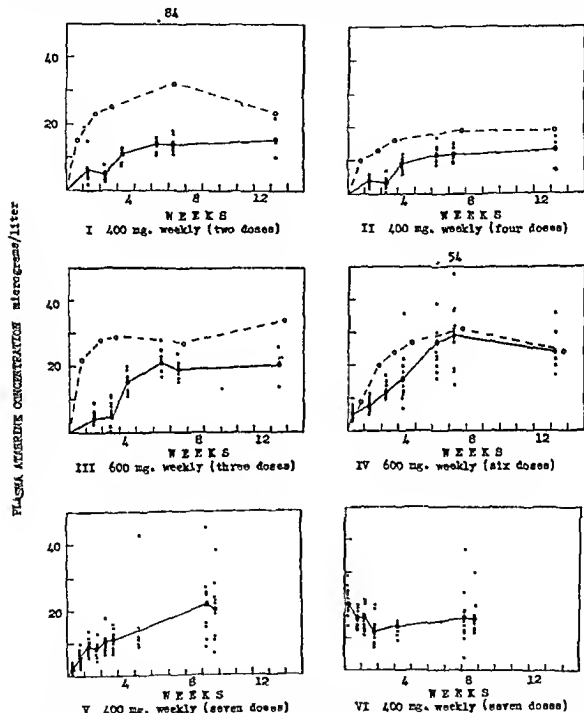
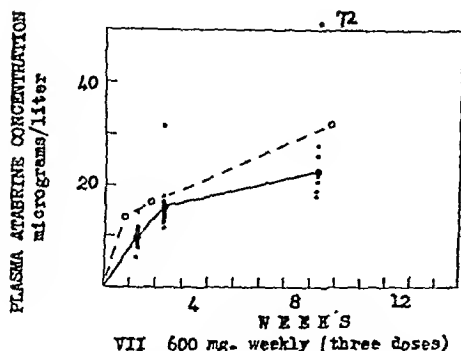


FIG. 1. WEEKLY MINIMAL AND MAXIMAL PLASMA ATABRINE CONCENTRATIONS OBSERVED DURING VARIOUS REGIMES OF SUPPRESSIVE ATABRINE THERAPY

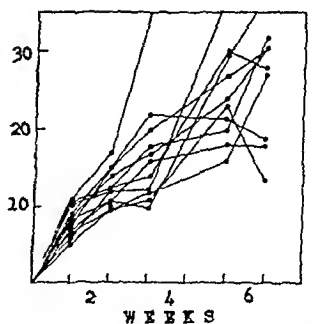
The mean-weekly minimum values by small dots. The mean-weekly maximum values by dashed line.

more and more a reflection of the minimal plasma atabrine concentrations observed.



VII 600 mg. weekly (three doses)

FIG. 2



IV 600 mg. weekly (six doses)

FIG. 3

FIG. 2. WEEKLY MINIMAL AND MAXIMAL PLASMA ATABRINE CONCENTRATIONS DURING THE ADMINISTRATION OF 600 MG. ATABRINE DIHYDROCHLORIDE WEEKLY

The symbols used are the same as in the case of figure 1. Refer to page 315 for the details of the regime of therapy and the relationship between the dosage schedule and the times of bleeding.

FIG. 3. THE WEEKLY MINIMAL PLASMA ATABRINE CONCENTRATIONS OBSERVED IN THE INDIVIDUALS OF A GROUP RECEIVING 600 MG. OF ATABRINE DIHYDROCHLORIDE WEEKLY IN SIX 100 MG. DOSES ADMINISTERED ON SIX CONSECUTIVE DAYS EACH WEEK (REGIME IV)

TABLE 5

Plasma atabrine concentrations

Observed in a series of subjects on regime 2; i.e., 100 mgm. atabrine dihydrochloride on successive days from Tuesday to Friday at 1 P.M.

WEEK.....	1			2			3			4			6			7			13		
DAY.....	Tu	Th	Fr	Tu	Th	Fr	Tu	Th	Fr	Tu	Th	Fr	Tu	Th	Fr	Tu	Th	Fr	Tu	Th	Fr
TIME.....	1P	1P	5P	1P	1P	5P	1P	1P	5P	1P	1P	5P	1P	1P	5P	1P	1P	5P	1P	1P	5P
SUBJECT	Plasma atabrine concentration (micrograms per liter)																				
Ben.....		4	7	4	12		2	10	17	8	11		9			9	18	17	13		17
Ber.....		5	11	6	10	16	7	13	16	10	13		12			11	14	20	7	19	18
Bey.....		5	15	5		15	4	12	20	10	12					15	19	28	17	20	23
Co.....		4	8	3		13	4	2		8	11		10			11	13	17	13	23	23
Gl.....		3	5	2	14	6	1	9	20	8	9		10			9	11	18	13	17	21
Do.....		5	8		14	15	2	13	24	11	15		17			14	14	15	15		
Cr.....		7	14	4	11	14	3		23	7	10		14			12					
Di.....		7	15	3	16	18	4	12		12	14		14			15					
Fe.....		2	13	5	7	10	2	7	12	6	10										
Er.....		4	5	2	13	11	0	7	13	8	12										
Mean.....		5	10	4	12	13	3	9	16	9	12		12			12	15	19	13	19	19

2. The absolute value of the mean plasma atabrine concentration finally achieved and maintained in each individual on suppressive atabrine is determined by the extent of the localization of atabrine in the body and by the balance which

is struck between the weekly dosage of atabrine and its rate of degradation in the body. This follows from the facts that absorption is essentially complete in the normal individual at these dosage levels and that renal excretion is negligible.

As is to be expected then, the total amount of atabrine administered each week is a more important determinant of the plasma atabrine concentration, and hence the amount of antimalarial protection, than the exact dosage schedule by which the drug is administered. Consequently, if it is found expedient to fix the routine for a group of individuals so that each receives a regular amount of atabrine each day, then this routine should be as effective as, but no more effective than, a regime which spaces larger individual doses of atabrine at less frequent intervals throughout the week.

3 The equilibrium plasma atabrine concentration which is characteristic of any dosage schedule may be achieved rapidly by the administration of a larger amount of atabrine over a relatively short period of time (regime 6).

It is difficult to evaluate the practical importance of this observation from the standpoint of the routine use of suppressive atabrine in an hyperendemic area. The incubation periods of the malaras are usually longer than 10 days and a certain amount of protection is conferred by any of the commonly used regimes of therapy during this interval. However, the observation is of undoubted importance when viewed in another light. Circumstances are frequently encountered where it is not possible to maintain continuity in the administration of atabrine for suppressive purposes. It is to be expected that the amount of protection will progressively diminish with the progressive lowering of the plasma atabrine concentration during a drug free interval. The data collected on regime 6 indicate that such individuals can be returned promptly to the point of maximal protection by a dosage schedule which administers additional atabrine at the time suppressive therapy is resumed. There can be no question of the practicality of this procedure in such a circumstance or of a similar procedure when suppressive atabrine is substituted for suppressive quinine. It must be assumed in each of these situations that the individuals concerned have malarial infections and that the time lag before the development of the clinical attack does not include an interval for the evolution of intermediate forms to the stage where trophozoites are released. The data on the rate of lowering of the plasma atabrine concentration during a drug free interval are meager. Such data, as are available, indicate that, although the rate is variable in the individuals of a group, it is generally sufficiently high for a considerable proportion of the group to have had a significant loss of protection within a week.

4 The data show a fairly wide spread in the plasma concentrations achieved in the individuals of each group.

This is particularly apparent in the group on regime 4 during the seventh week. The mean minimal plasma atabrine concentration is 29 micrograms per liter during this week. However, the spread in the individual determinations is from 14 to 48 micrograms per liter.

Discussion Present suppressive atabrine therapy leaves much to be desired when applied to groups of wholly susceptible individuals in hyperendemic

malarious areas. The occurrence of clinical malaria in an individual under these conditions may be related to the presence of a strain of plasmodia which is unusually resistant to the action of atabrine, or, to a plasma atabrine concentration which is insufficient to prevent the exuberant growth of the ordinary strains of plasmodia in a given geographical area. The correlation between antimalarial activity and plasma atabrine concentration is such that the individuals of a group with the lower plasma concentrations may be assumed to have a higher susceptibility to clinical malaria than the remainder under comparable conditions of exposure. The data presented above indicate that a fair proportion of any group on a regime of suppressive therapy will have plasma atabrine concentrations considerably below the mean of the group as a whole. The omission of individual doses or the presence of gastro-intestinal disturbances which may interfere with absorption may be expected to increase proportionately the number in a group who fall in the lower range of concentration.

Clinical malaria, which develops subsequent to the withdrawal of an individual from an hyperendemic area and the cessation of atabrine therapy, may also be related in part to the plasma atabrine concentration during an exposure and that subsequently maintained. Or again, it may be more intimately related to the characteristics of the infectious agent, or, to the frequency and size of sporozoite dosage.

A decision on the relative importance of each of these factors in determining the attack rates of the malarias cannot be made with our present information. Other studies are obviously indicated. The distribution of plasma atabrine concentrations which are achieved in individuals on several dosage schedules of suppressive atabrine therapy should be described statistically in relatively large groups. These groups should be composed of individuals leading a highly active life under the environmental conditions which obtain in tropical and subtropical climates.⁴ Such data may then serve as a background for other studies which determine the plasma atabrine concentrations of individuals on suppressive therapy at the time of appearance of clinical malaria. It must also be established whether there is a relationship between the plasma atabrine concentration during and subsequent to an exposure to infected mosquitoes and the development of clinical activity subsequent to the termination of suppressive therapy. It should be possible with these items of information to define the relative importance of each factor which contributes to the establishment of malarial infections during and following suppressive therapy. It may also be possible to construct regimes of atabrine therapy which will yield reasonably predictable attack rates for a given geographical area.

Meanwhile, it may be assumed with some confidence, that the concurrent

⁴ These observations should be controlled by a cross-sectional study of the plasma atabrine concentrations achieved on a similar regime of suppressive therapy during continuous exposure to malaria. It may be assumed that a considerable number of individuals in such a group will have contracted a malarial infection though there are no signs of clinical activity. However, the tissue response to the infection may influence the degree to which atabrine is localized, or, the rate at which it is degraded to an extent which will alter the relationship between oral dosage and plasma atabrine concentration.

protection afforded an individual by suppressive therapy is proportional to the plasma atabrine concentration maintained. It may similarly be assumed, but with less certainty, that the ultimate protection is also determined in part by the concentration of plasma atabrine at the time of the inoculation of sporozoites or during the first few days of an infection, and, in part by the concentration achieved and maintained thereafter. There is much to be said, in this view, for a regime of suppressive therapy which confers the maximal protection from the first day of exposure to infected mosquitoes. The general characteristics of the metabolism of atabrine are such that this condition can be satisfied if suppressive therapy is initiated some weeks before entering an hyperendemic area or if large doses of atabrine are administered just before or during the initial days of the exposure.

SECTION III. AN EXAMINATION OF SEVERAL REGIMES OF ATABRINE THERAPY WHICH MAY BE USED IN THE TREATMENT OF CLINICAL MALARIA. The observations summarized in this section describe the relationship between a variety of dosage regimes of atabrine which may be used in the treatment of clinical malaria and the plasma atabrine concentrations commonly achieved. It is possible through these data to utilize the general information on the physiological disposition of atabrine previously presented in establishing the more important principles upon which rational atabrine therapy must rest.

Experimental Methods. The chemical method used for the estimation of plasma atabrine concentrations was the same as for those of Section II. It is of practical importance to appreciate that the method is relatively simple when the plasma atabrine concentration is in the range usually encountered in the treatment of the clinical attack.

Clinical Material. Patients were used who had just completed or were completing a course of therapeutic malaria. The activity of the malaria in each case is rated as ++, +, or 0, depending on whether at the time atabrine was given, the patient had fever and parasitemia, parasitemia but no fever, or no fever and no parasitemia.

This type of clinical material is not suited to test the therapeutic efficacy of the several dosage regimes examined. However, the individuals may be expected to have undergone a tissue response to the malarial infection similar to that which occurs in the naturally acquired disease. This precaution was taken since the tissue response to a malarial infection may affect the extent of the localization of atabrine or its rate of degradation.

Experimental results. Present routine therapy. The plasma atabrine concentrations observed in ten consecutive patients receiving 0.1 g atabrine dihydrochloride three times daily are summarized in table 6. These plasma atabrine concentrations may be taken as typical of those commonly achieved during the usual regime of atabrine therapy (2). It should be noted that the plasma atabrine concentrations are generally low during the initial days of therapy although they subsequently increase. However, there is considerable variation in the rate of the increase of plasma atabrine concentration and in the concentration attained by the fifth to the seventh day. There is no correlation between plasma atabrine concentration and body weight. The latter finding is true for the other regimes studied.

Other regimes of therapy. The obvious inadequacies of this regime of therapy (see discussion) led to the investigation of others constructed in accord with more

rational therapeutic principles. All regimes have one feature in common. They are designed to achieve a high plasma atabrine concentration on the initial day of therapy and to sustain this throughout succeeding days. The number of patients used in each series is small but is adequate for the general purposes of this study.

Intravenous atabrine: The intravenous administration of atabrine was first examined to determine whether this procedure is feasible as a routine measure to insure a high initial plasma atabrine concentration. The whole blood atabrine concentrations were observed in a series of patients following the intravenous administration of 0.4 to 1.0 gm. of the dihydrochloride. The chemical method

TABLE 6

Plasma atabrine concentration during the oral administration of 0.1 gm. atabrine dihydrochloride three times daily

These observations were obtained in a consecutive series of patients during the termination of an attack of induced malaria. Each received 0.1 gram atabrine dihydrochloride three times daily after meals, the times being 7:00 A.M., 11:30 A.M., and 5:00 P.M. The estimation of atabrine in the plasma was at 24 hour intervals. The blood sample being drawn at 11:00 A.M. Detailed information indicates that the plasma atabrine concentration at the latter time is a fair reflection of the mean concentration during each 24 hour interval.

NO.	PT.	WEIGHT	ACTIVITY OF MALARIA	DAYS OF THERAPY	PLASMA ATABRINE CONCENTRATION (MICROGRAMS PER LITER)					
					Days after beginning therapy					
					1	2	3	4	5	6
		kg.								
1	Ni	74	++	6	17	27	30		29	
2	Ca	60	++	6	15	38	59	44		57
3	Na	45	++	6	20	43	74	53	67	80
4	Vi	57	++	6	12	27	29	40	75	56
5	Be	75	++	6	24	35	29	43	69	51
6	Sh	57	++	6	15	30	31		35	
7	Me	56	+	7	4		34	68	102	94
8	Pr	77	0	7	11	20	18	20	30	37
9	He	70	++	8	23	30	36	40	33	43
10	Si	63	++	8	5		16	28	37	36

was not refined sufficiently, at the time, for application to the measurement of atabrine in plasma. However, some of the data are presented in table 7 since rather severe toxic reactions were encountered which precluded further investigations of this type.

The reactions observed in patients Wo and Wa were characterized by a profound depression in the rate of respiration. In addition, Wo experienced a mild convulsive-like episode. The whole blood atabrine concentrations at which these occurred were in the order of 0.7 to 0.9 mg. per liter. These reactions occurred at dosage levels of 0.77 and 0.81 gm. It appears from the remainder of the data that amounts of atabrine dihydrochloride up to and including 0.4 gm.

may be administered intravenously to the average adult without adverse effects, providing the rate of administration is slow. However, the procedure is not advised as a routine measure.

Combined intramuscular and oral atabrine One group of patients received an initial intramuscular injection of 0.4 gm of atabrine dihydrochloride together with an oral dose of 0.1 gm at 10 P.M. Oral doses, spaced at varying intervals were then given for a varying number of days. Data from a series of these patients are summarized in table 8. A high plasma atabrine concentration which

TABLE 7

Whole blood concentration of atabrine following the intravenous infusion of atabrine dihydrochloride

0.5 gram of atabrine dihydrochloride was initially administered intravenously by syringe (30 minutes), the remainder was then run in by slow infusion. The rate of atabrine administration is calculated as the mean rate for the total dosage.

PATIENT	Ba	Ml	Gr	Wo	Ch	Wa
Weight (kg)	60	63	82	40	56	65
Atabrine administered rapidly (g) slowly (g)	0.5 0.5	0.5 0.31	0.5 0.26	0.5 0.27	0.5 0.31	0.5 0.31
Total dose (g) mg/kg mg/kg/min	1.0 16.7 0.060	0.81 12.9 0.086	0.76 9.3 0.052	0.77 19.2 0.137	0.81 14.3 0.88	0.81 12.3 0.083
Time for administration (minutes)	280	150	180	140	165	150
Time	Whole blood atabrine concentrations (micrograms per liter)					
20 minutes	690	920	640	830*	940*	1,090
1 hour	440	690				
3 hours		650	390	760*	690*	790
1 day	274	248	171	425	236	214
2 days	143	201	159	217	205	111
3 days	141					
4 days		114	106	166	138	82
5 days	112					
6 days						
7 days		117	59	106	97	50
8 days	57					

* Severe toxic symptoms at this time

is consistently reached within three hours after the injection may be taken to indicate that atabrine administered by this route is rapidly absorbed. As is to be expected the plasma atabrine concentration during subsequent days reflects the size and frequency of the maintenance dose to an ever increasing extent. However, it is apparent from these and other data that 0.1 gm atabrine dihydrochloride three times daily will not usually maintain a plasma atabrine concentration in excess of that which is achieved following the intramuscular injection of 0.4 gm of the drug.

Oral atabrine: A regime of therapy which includes an intramuscular injection may not be generally practical. Consequently, other regimes were examined which are designed to produce a high initial plasma atabrine concentration by the oral administration of atabrine. In the first of these (table 9), 0.2 gm. of the hydrochloride is administered each six hours for the first day, thereafter 0.1 gm. is administered at eight hour intervals. The large oral doses do not systematically produce as high a plasma atabrine concentration during the initial hours of therapy as when a portion of the atabrine is administered intramuscularly

TABLE 8

Plasma atabrine concentration during the therapeutic administration of atabrine dihydrochloride by combined intramuscular and oral routes

These observations were obtained in a series of patients during the termination of an attack of induced malaria. Each received an initial intramuscular injection of 0.4 gram and an oral dose of 0.1 gram atabrine dihydrochloride at 10:00 P.M. followed by 0.3 gram orally distributed throughout the next 12 hours. The atabrine was administered in 0.1 gram doses on the subsequent days after meals at 7:00 A.M. and 5 P.M. when 0.2 gram were given and at 7:00 A.M. and 11:30 A.M., and 5:00 P.M. when three doses were given daily. The estimation of plasma concentration was three hours after the initial intramuscular injection and daily at 11:00 A.M. thereafter.

PT.	WEIGHT	ACTIVITY OF MALARIA	INITIAL I.M. INJ.	THERAPEUTIC ATABRINE REGIME ORAL DOSES (GRAMS)						PLASMA ATABRINE CONCENTRATION (MICROGRAMS PER LITER)							
										Time after intramuscular injection							
				1	2	3	4	5	6	3 hrs.	9-11 hrs.	1 day	2 days	3 days	4 days	5 days	6 days
	kg.																
McQ.....	63	+	0.4	0.3	0.2	0.2	0.2	0.2		155		70	60	64	78	59	
D'A.....	70	+	0.4	0.3	0.2	0.2	0.2	0.2		100		38	42	29	33	41	
Kr.....	70	++	0.4	0.3	0.3	0.2	0.2	0.1		78		44	35	51			
Ju.....	50	++	0.4	0.4	0.3	0.2	0.2	0.2	0.1	111	126		100	75	67	55	44
Fu.....	60	++	0.4	0.4	0.3	0.2	0.2	0.2	0.1	51	39	34	40	51	56	47	
Es.....	74	++	0.4	0.4	0.2	0.2	0.2	0.2	0.1	94	72	121	121	115	135	143	109
McB.....	60	++	0.4	0.4	0.3	0.3	0.3	0.3	0.1	82	63	41	47	48	50	43	35
Bi.....	60	++	0.4	0.4	0.3	0.3	0.3	0.3	0.1	138	128	96	114	104	90	101	81
St.....	57	++	0.4	0.4	0.3	0.3	0.3	0.3	0.1	80	78	61	71	55	70	92	80
Ke.....	70	++	0.4	0.4	0.3	0.3	0.3	0.3	0.1	78	65	54	67	53	52	53	37
Sc.....	78	++	0.4	0.4	0.3	0.3	0.3	0.3	0.1	74	49	43	57	73	61	74	50
Cu.....	54	++	0.4	0.4	0.3	0.3	0.3	0.3	0.1	68	44	44	59	88	78		

although the groups are too small for a precise comparison. However, the plasma atabrine concentrations are of the same order at the end of 24 hours; on the regime of oral therapy varying from 19 to 124 micrograms per liter whereas the variation in the group receiving intramuscular atabrine is from 34 to 121. The subsequent course of the plasma curves indicate that a low value at 24 hours with the intramuscular atabrine is a reflection of a higher than usual rate of degradation while the low plasma concentrations observed at this time in some of the patients on exclusively oral therapy may be in part a reflection of the less com-

plete absorption of the atabrine at the high dosage level (c.f. table 4). Again, the plasma atabrine concentrations during the later days of the regime are dominated to an increasing extent by the maintenance dosage. The plasma levels of the individuals of the group vary widely although the group as a whole tends to stabilize after several days in the range of 50-100 micrograms per liter.

Another regime of therapy examined consists of the oral administration of 0.2 gm. atabrine dihydrochloride at six hour intervals for two successive days and

TABLE 0

Plasma atabrine concentration during the oral administration of atabrine by regimes which are designed to produce high initial plasma concentrations

These observations were obtained in a series of patients during the termination of an attack of induced malaria. The dosage regime administered 0.8 grams atabrine dihydrochloride on one day followed by 0.1 gram three times daily thereafter. The large initial doses were administered in 0.2 gram doses after each of the three daily meals with an additional dose between 8:00 and 9:00 P.M. The sustaining 0.1 gram doses were also administered after the daily meals at 7:00 A.M., 11:30 A.M., and 5:00 P.M. The estimation of atabrine concentration in the plasma was at 24 hour intervals, the blood samples being drawn at 11:00 A.M.

PATIENT	WEIGHT	ACTIVITY OF MALARIA	DAYS ON ATABRINE REGIME		PLASMA ATABRINE CONCENTRATION (MICROGRAMS/LITER)						
			0.8 g daily	0.3 g daily	Days after beginning therapy						
					1	2	3	4	5	6	7
Dav	61	+	1	5	53	76	56	64	64	53	
Dal	52	+	1	5	70	50	61	71	55	80	
Den	76	+	1	5	35	43	66	67	68	65	
Ei	70	+	1	5	41	58	58	76	76	61	
Va	59	+	1	5	66	57	75	78	70	64	
Sh	46	++	1	5	49	52	60	82	109	78	50
Ca	59	0	1	6	48	25	28	29	40	41	42
Co	102	+	1	6	52	58	45	60	52	69	87
DeB	47	+	1	6	124	120	97	114	160	121	98
Ga	59	+	1	6	75	60	67	72	70	56	55
Ha	49	+	1	6	44	65	63	57	62	49	
Ka	68	+	1	6	83	99	40	58	58	58	65
Pi	57	0	1	6	66	73	96	89	92	41	45
Ri	99	0	1	6	19	27	44	36	33	25	38
Yo	57	0	1	6	90	109	102	87	67	61	64
Zc	85	++	1	6	44	41	48	43	52	82	45

0.1 gm three times daily thereafter for an extended period of time. These observations were designed to determine whether two days of high dosage produce more uniformly high plasma concentrations during the early days of therapy than when a high dosage is given for a single day; also whether such a high dosage is accompanied by a significant number of gastro-intestinal reactions. The administration of the maintenance doses for a longer period of time was designed to determine whether an equilibrium between oral dosage and plasma concentra-

tion is usually achieved on such a regime by the seventh day or whether the continued administration of atabrine at this dosage level is accompanied by a further increase in plasma level and perhaps toxic reactions of a systemic nature. These observations are summarized in table 10.

High initial plasma concentrations are the rule with this regime and most patients accept the large doses without serious gastro-intestinal disturbances. The plasma atabrine concentration is stabilized well before the seventh day and no increases were seen with a continuation of the maintenance dosage. The plasma concentrations observed were in the same range as in the case of the

TABLE 10

Plasma atabrine concentration during the oral administration of atabrine by regimes which are designed to produce high initial plasma concentrations

These observations were obtained in a series of patients during the termination of an attack of induced malaria. The dosage regime administered 0.8 gram atabrine dihydrochloride on each of two consecutive days followed by 0.1 gram daily thereafter. The large initial doses were administered in 0.2 gram doses after each of the three daily meals with an additional dose given between 8:00 and 9:00 P.M. The sustaining 0.1 gram doses were also administered after the daily meals at 7:00 A.M., 11:30 A.M., and 5:00 P.M. The estimation of atabrine concentration in the plasma was at 24 hour intervals, the blood samples being drawn at 11:00 A.M. each day.

PT.	WT. kg.	ACTIVITY OF MALARIA	DAYS ON ATABRINE REGIME		PLASMA ATABRINE CONCENTRATION (MICROGRAMS/LITER)													
			0.8 gm. daily	0.3 gm. daily	* Days after beginning therapy													
					1	2	3	4	5	6	7	8	9	10	11	12	13	14
We.....	72	++	2	12	87	140	127	108	130									120
Ro.....	64	+	2	12	140	171	128	98		105	109	89		113				104
DeC.....	57	+	2	12	43	62	62	63	50	80	61	63	73	107	75			
Ta.....	71	+	2	12	53	93	60	65	87	87	94	92	83	120	88			
As.....	79	++	2	12	49	47	52	73	66	70	57	84	62	44	52	46	60	64
Bo.....	62	+	2	12	90	61	105	110	101	96	99	90	108	80	100	103	98	100
Mo.....	70	+	2	12	59	86	72	60	70	77	60	74	75	86	80	83	124	83
Wo.....	49	+	2	12	91	152	110	147	96	98	90	90	103	97	114	96	120	180
Qu.....	51	0	2	12	47	64	57	46	55	58	61	66	64	67	78	83	73	90
Pa.....	50	++	2	12	75	152	135	105	108	100	108	97	140	119	135	127	110	87
Ke.....	61	++	2	12	73	134	108	104	97	83	70	70	78	120	79	83	83	73

previous regime (table 9) but generally somewhat higher. No significance may be attached to the difference in view of the variation observed in each group and the number of subjects studied.

The remainder of the regimes examined do not warrant special presentation. It was demonstrated that adequate plasma atabrine concentrations can be maintained for one or several days by the exclusive use of parenteral atabrine. The plasma atabrine concentrations though generally higher are in the same range as when similar amounts are given orally. Following an initial intramuscular injection of 0.4 gm. atabrine dihydrochloride serial injections of 0.2 grams

may be given for two or three doses at eight hour intervals and continued at 12 hour intervals until oral administration is possible. Extended parenteral atabrine therapy is rarely warranted. Other observations indicate that an elevation of the daily maintenance dose from 0.3 to 0.4 gm atabrine dihydrochloride or higher is accompanied by a roughly comparable elevation of the range of plasma atabrine concentrations. An insufficient number of patients have been observed on dosage regimes in excess of 0.4 gm daily to be certain how high the daily maintenance dose may be placed with safety.

Toxic Manifestations Particular attention was paid to the presence or absence of adverse reactions which could be attributed to the larger oral doses of atabrine or to the high plasma atabrine concentrations. A few minor reactions were observed. However, none was sufficiently severe to require either the withdrawal of the drug or a serious modification of the dosage regime. The data on the individuals showing adverse gastro intestinal reactions do not indicate that the reactions seriously modified the plasma atabrine concentrations achieved although brisk diarrheas may be expected to do so by limiting the completeness of absorption from the gastro intestinal tract. Several psychoses were encountered. It is difficult to be certain that these were due to the atabrine alone since each patient had some neuro psychiatric changes due to the underlying CNS syphilis.

The acute toxic reactions encountered with intravenous atabrine deserve some special comment. The amount of atabrine per kilo of body weight as well as the rate of administration was low in each of these cases as compared to the amount and rate required to produce a toxic reaction in the dog (10). The severity of the reactions, together with the latter fact may indicate a specific susceptibility on the part of the human subject. It is logical to suppose that such a susceptibility is related, at least in part, to the slower and less extensive localization of atabrine in the human subject (Section I). In any case, the observations indicate that the intravenous administration of atabrine is not advisable as a routine measure in the treatment of human malaria. It should be noted that whole blood atabrine concentrations, in the range which obtained during these toxic manifestations, are frequently exceeded without adverse effects when the atabrine is administered orally over a period of days. This is not a surprising finding. The plasma atabrine concentration during and just following an intravenous injection of atabrine may be expected to be disproportionately high as compared to that at a similar whole blood concentration when the atabrine is given in a more conventional manner.

Discussion The data, in general, reflect the properties of atabrine which result in its extensive localization in specific organs. This feature of the physiological disposition of atabrine together with low rates of degradation and excretion lead to the retention of atabrine in the body. An appreciation of these general characteristics is important in viewing antimalarial therapy in relation to the ends which such therapy should satisfy. Briefly, these are a prompt recession of clinical activity and a final termination of the infection.

An acute recession of the clinical manifestations of malaria is not to be expected

with the usual regime of therapy (i.e., 0.1 gm. atabrine dihydrochloride three times daily). This follows from the low plasma atabrine concentrations which are so frequently observed during the initial days on this regime. The delay in the therapeutic response has led to the general use of a preliminary two or three day course of quinine. Atabrine was then administered for a five day period in the usual dosage (11). The latter procedure generally produces the desired acute recession of clinical attack. However, such combined therapy has not proven altogether satisfactory. It is to be expected that on this regime a small proportion of patients will not attain a sufficient concentration of quinine in the plasma to produce a prompt cessation of clinical activity (5). More important, perhaps, is that a fair proportion of individuals will only attain low plasma atabrine concentrations by the fifth day. It is unlikely that the latter patients will receive the maximal therapeutic benefit which is to be derived from the atabrine. The high proportion of short term recurrences which have been encountered with this regime of combined therapy may result in part from the latter circumstance. It is logical to suppose, from these considerations, that such combined therapy is not certain to achieve either of the two ends which are so desirable in all antimalarial therapy.

Rational regimes of antimalarial therapy should be designed along commonly accepted principles of chemotherapy. The latter are well exemplified by the present usage of the sulfonamides in the treatment of acute infections, i.e., sufficient drug is administered on the diagnosis of a disease to obtain the desired blood concentration and the latter is then maintained by the serial administration of smaller doses. These principles are related in a simple fashion to the use of atabrine by the data presented in tables 8, 9, and 10. It is suggested that a regime of the general type which administers 0.8 gm. of atabrine dihydrochloride on each of one or two days and 0.3 gm. each day thereafter has much to recommend it as a therapeutic procedure. However, such a dosage schedule may be considered to administer the minimal rather than a maximal amount of drug. The amount required or rather the plasma concentration which is necessary to obtain the maximal therapeutic benefit of atabrine as well as the duration such a concentration must be maintained can only be determined by quantitative studies on the naturally acquired disease in wholly susceptible patients. These studies must take into account that the duration of the therapeutic effect extends well beyond the time during which the drug is administered.

The experience of this Service indicates that most patients in fair physical condition will tolerate the oral administration of 0.8 to 1.0 gm. of atabrine dihydrochloride in 24 hours without undue distress. However, the parenteral administration of a portion of this initial amount is frequently advisable. Dysentery is a fairly common concomitant infection in patients with malaria. Also, a small proportion of individuals on high oral doses of atabrine may be expected to develop a diarrhea. Either of these complicating factors may interfere with the absorption of the drug with a consequent loss in therapeutic benefit. This dual uncertainty argues against the use of an exclusively oral route in patients where an immediate chemotherapeutic effect is required. It is suggested that

0.4 gm of atabrine dihydrochloride be administered intramuscularly to such patients on the diagnosis of the disease and that 0.1 gram of atabrine be administered orally at that time and at not less than six hour intervals during the first 24 hours and at not less than eight hour intervals thereafter. Such a regime will assure an initial plasma atabrine concentration which should be adequate to produce an acute recession of the disease and this concentration will be approximately maintained during the subsequent days of therapy. It is possible to use an exclusively parenteral route for the administration of atabrine when necessary, although this is rarely necessary.

The parenteral administration of atabrine has fallen into disrepute despite a general acceptance that intramuscular atabrine produces a most acute termination of clinical malaria (2). The reasons are quite specific and while they do not bear directly on our present problem they warrant some mention. The majority of clinicians, who have supported this form of therapy, have utilized it exclusively rather than as an adjunct to the oral administration of the drug because of specific indications. A second objection has arisen from the local irritation which is caused by the intramuscular injection of atabrine musonate (diethyl sulfonate).

It has been the experience of this Service that the intramuscular injection of the dihydrochloride is not attended by sufficient local discomfort to preclude this form of medication.

The above discussion relates more to the treatment of the usual patient with a malarial infection than to the patient who presents a specific therapeutic problem. The latter category includes individuals who manifest breakthroughs of clinical activity while receiving what is generally adequate suppressive therapy for a given geographical area, those who do not respond to therapy with a prompt disappearance of paroxysms and parasites, and those who manifest a recrudescence of clinical activity shortly after the termination of therapy.

The factors which may be involved in a breakthrough of clinical activity while on suppressive atabrine therapy have been mentioned in Section II. They require some repetition, in relation to subject material of the present section, because of their direct bearing on the problems of definitive therapy. The failure of suppressive therapy in a given individual may be accepted as a specific indication that the clinical attack in this individual constitutes a therapeutic problem. It must be presumed, providing it is known that he has received atabrine in amounts which are adequate to prevent clinical malaria in a group as a whole, that he departs from the usual because of the characteristics of the offending plasmodium or because of physiological considerations which limit the effectiveness of the administered atabrine. Among the latter considerations are the absorption, localization, degradation, and excretion of atabrine which together with the dosage schedule determine the plasma atabrine concentration on any regime of therapy. The sum of these separate physiological processes may be such in an individual, that the plasma atabrine concentration attained is compatible with the exuberant growth of a given strain of plasmodia. It is to be expected that the same factors will operate to produce a lower than usual plasma atabrine concentration when full therapeutic doses of the drug are administered.

It would be hazardous, in such a situation or when an infection is due to an atabrine resistant strain of plasmodium, to expect the usual clinical effect from any given dosage regime of atabrine.

SUMMARY

The important factors which are concerned with the physiological disposition of atabrine have been defined.

The distribution of atabrine in the blood is such that observations on its specific antimalarial action should be related to the concentration of the drug in the plasma and, perhaps, indirectly from this, to its concentration in plasma water. The plasma atabrine concentration achieved after single or serial doses is dominated by the tendency of the organs of the body to localize the material within them and by the slow rate at which the drug is degraded. These characteristics are reflected in the low plasma atabrine concentration which is reached after a single dose of atabrine as well as in the low rate of renal excretion. They also, together with the low excretion rate, are reflected in the slow rate of fall of the plasma atabrine concentration on the termination of therapy and by the progressive accumulation of the drug in the body when serial doses are administered over a period of days or weeks.

The administration of repeated doses eventually results in the attainment of an equilibrium between the amount of drug administered and its localization, degradation and excretion. Thereafter, fairly constant plasma atabrine concentrations are maintained with a continuation of the dosage schedule.

The practical importance of the above factors has been examined by a study of the plasma atabrine concentrations achieved in groups of individuals on various regimes of suppressive and definitive therapy.

Acknowledgment. We wish to acknowledge, with thanks, the assistance of Dr. George B. Wallace, Professor of Pharmacology, New York University College of Medicine, in arranging and carrying out the study of suppressive therapy on the group of medical students.

REFERENCES

- (1) Circular Letter No. 153, Office of the Surgeon General, War Department, J. A. M. A., 123: 205, 1943.
- (2) The Treatment of Malaria, Fourth general report of The Malarial Commission and appendixes. Bulletin of the Health Organization, League of Nations, 6: No. 6, December 1937.
- (3) MASEN, J. M., J. Biol. Chem., 148: 529, 1943.
- (4) BRODIE, B. B., AND UDENFRIEND, S., J. Biol. Chem., 151: 299, 1943.
- (5) In preparation.
- (6) DEARBORN, E. H., KELSEY, F. E., OLPHAM, F. K., AND GEILING, E. M. K., THIS JOURNAL, 78: 120, 1943.
- (7) CRAIG, L. C., J. Biol. Chem., 150, 33, 1943.
- (8) KELSEY, ET AL, To be completed in proof.
- (9) SHANNON, J. A., BRODIE, B. B., AND MILLER, H. A., Report No. 6, Subcommittee on The Coordination of Malarial Studies, Division of Medical Sciences, National Research Council. June 8, 1943.
- (10) MOLITOR, H., and UNA, K., Personal Communication.
- (11) Circular Letter No. 56, Office of the Surgeon General, War Department, War Medicine, 1, 539, 1941.

IODINE IN BLOOD AND THYROID

VII AN ANALYTICAL PROCEDURE FOR USE WITH SMALL SAMPLES PHARMACOLOGICAL RANGE OF CONCENTRATIONS

T S SAPPINGTON,* N HALPERIN, AND W T SALTER

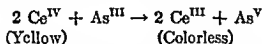
From the Laboratories of Pharmacology and Toxicology, Yale University School of Medicine

Received for publication April 27, 1944

In studies of the metabolism of iodine, particularly in relation to endocrine function, it has become clear (1, 2, 3) that the function of the thyroid gland can be measured with considerable confidence by the newer methods of micro-analysis for iodine. Unfortunately, at the present time, the best methods available require 6 to 10 cc of serum or plasma. This amount, although a great improvement on former methods, nevertheless limits seriously the use of iodine analysis. This is true because duplicate analyses are essential and it is difficult to obtain the requisite amounts of 25 to 40 cc of whole blood repeatedly from even an adult patient, and also because the methods are useless for experimentation with small animals. In consequence, despite the demonstration (2) that the function of the thyroid can be measured satisfactorily by blood analysis, the method is not generally used either in the clinical or in the experimental laboratory.

In order to implement our present knowledge and make it available for general use it will be necessary to devise methods which employ amounts of serum of the order of 1 cubic centimeter or less. It is the purpose of this paper to describe such a method, adapted to higher concentrations of iodine such as are encountered in the use of therapeutic agents containing iodine. A companion paper, published elsewhere, will describe a modification devised to cover the more "physiological" range of normal and abnormal thyroid function (4).

The method employed was suggested by Chancy (5) and based on the work of Sandell and Kolthoff (6) who studied the action of iodine as a catalyst upon the following reaction



In the course of this reaction the yellow ceric ion changes to the colorless cerous ion. This change can be measured in a photoelectric colorimeter such as the Klett-Summerson (7). If the mixture is kept at a standard temperature, the speed of the reaction will be a function of the iodide concentration present in the solution.

ANALYTICAL PROCEDURE The method used was a modification of the techniques employed by Kendall (8), Perkin (9) and Groák (10), combined with the

* Research Fellow in Pharmacology

Aided by a grant from the Fluid Research Fund of the Yale University School of Medicine

method of Chaney (5). These techniques have been modified considerably so that the whole method as it is now described should be considered as an entity without blind attempts to substitute the technical details of the original authors.

Preparation and oxidation of organic materials. Before the iodine in thyroid or serum can be determined as iodide, the organic matter must be ashed. Prior to ashing, it is essential to reduce the sample to a homogeneous liquid. In so doing, it is necessary to bear in mind the ultimate concentration of iodide which will be attained. This problem is approached as follows.

Determination of thyroid iodine. One milligram of fresh normal thyroid contains approximately 0.4 microgram of iodine. This amount corresponds to the middle of the range of samples under discussion. The amount of fresh gland represented in this one-milligram sample would be readily available in most experiments involving small animals.

The dissected glandules are kept in a moist chamber preparatory to weighing. They are then transferred to a previously tared tiny glass cup which is in readiness on the balance pan. After weighing the tissue is placed in a tube graduated at 5.0 cc. with 1.0 cc. of 2.0 N sodium hydroxide. By triturating, rubbing, stirring and warming a homogeneous near-solution is obtained. The volume is finally made up to the 5.0 cc. mark, and appropriate samples measured out for oxidation and analysis as described later.

Characteristic values for the thyroid of an adult male rat were as follows: Fresh tissue weight, 11.4 mgm. Iodine in 0.5 cc. sample (one-tenth of total) was 0.427 microgram. Total iodine in the thyroid tissue 4.27 micrograms. Concentration in fresh thyroid 0.038 per cent.

Determination of Serum Iodine. Serum is preferable to plasma because its electrolyte content will not have been altered by anticoagulants. The serum is oxidized directly as described below, without preliminary treatment. The usual sample is 0.5 to 1.0 cc., accurately measured. In Table I are given representative determinations of the iodide (a) in two lots of pooled human serum and (b) in the serum of a single patient. In both instances the concentration of total iodine in the serum was determined first and then the concentration was measured after reinforcement of the serum with known amounts of iodide. It will be observed that a standard error of less than ten per cent is to be expected for averaged duplicates and this is sufficient for most clinical and biological purposes. The accuracy can be improved by more careful thermostatic control of the temperature at which the reaction takes place, but it is debatable whether this refinement is needed.

Combustion of Organic Materials. Into a pyrex test tube (of approximately 1 cm. internal diameter and 10 cm. length) is placed 2.0 millimols, i.e., 212 milligrams, of anhydrous sodium carbonate (iodine-free) and a small amount of water sufficient to dissolve the carbonate. To this solution is added 1 cc. of serum, or an appropriate sample of thyroid extract. The tube is then clamped in an inclined position so as to rest in warm water at about 50°C. and a blast of air is sucked through a capillary over the surface of the solution. As the liquid evaporates, the tube is rotated occasionally in order that the dried material may

TABLE I
Total iodine in pooled serum*

POOLED SERUM #1	KI ADDITION	IODINE FOUND	IODINE EXPECTED
cc	I in $\mu\text{e} \times 100$	$\mu\text{e} \times 100$	$\mu\text{e} \times 100$
0.5		13	
0.9	0	30	
	0	29	
	0	32	
	0	24	
Av		29	
0.9	10	44	39
0.9	26	59	
		57	
Av		58	55
0.0	40	56	
		69	
Av		68	50
POOLED SERUM #2			
1.00		93	01
0.75		78	68
0.50		47	46
0.25		19	
		22	23
0.10		8	9
0.05		5	5

Total iodine in a single human serum†

0.0	0	4.5	4.4
0.0	0	3.0	4.4
0.9	0	6.0	4.4
0.9	20	29	24
0.9	10	16	14

* This pooled serum was obtained at random from various patients whose iodine intake, dietary or medicinal, was not controlled

† By Riggs Man (15) method 4.0

be smeared evenly about the inside of the test tube. The tube is next placed in an electric muffle furnace. (A convenient apparatus is that manufactured by the Will Corporation, Rochester, New York, ranging up to 1100°C.)

During the initial stages of the heating care must be taken not to heat too fast, else entrapped steam will explode and blow the contents of the tube out into the furnace. Once the material is sufficiently dried, however, the temperature can be elevated rapidly in the course of thirty minutes up to exactly 600°C . After thirty minutes the temperature is lowered to 550°C ., and is maintained at this level for two hours longer. The oven is then allowed to cool rapidly and the tube removed. With materials low in iodine, the contents of the tube may be dissolved by trituration in 1.9 cc. of distilled water, to which is added subsequently with caution 2.0 cc. of 7 Normal sulfuric acid.

Next, a suitable aliquot (e.g., 1.5 cc.) of the contents of the digestion tube is transferred to a tube graduated at 5 cc. Enough additional sodium carbonate is added to bring the total contents of the tube to one millimol or 106 milligrams

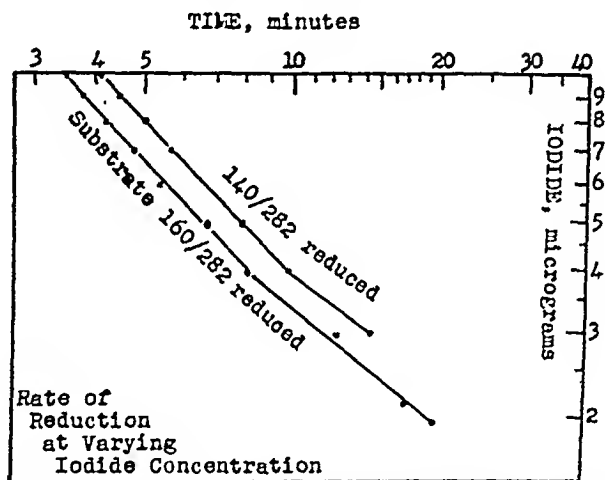


FIG. 1. By measuring the time required to reduce a certain fraction of the substrate, the iodide concentration can be estimated, if other variables are appropriately controlled.

of sodium carbonate. Thus the total content of sodium will be 4.0 milliequivalents. Enough acid is added to bring the total present up to 1 cc. of 7 M. Then distilled water is added to the 5.0 cc. mark. After thorough mixing of the solution, a 3.0-cc. sample is pipetted into a Klett-Summerson colorimeter tube. Then the standard amount of ceric solution is added and the reaction is initiated by adding arsenious acid at zero time followed by enough water to adjust the volume. It is well to use a known standard solution in association with several unknown solutions as a check on the colorimetric procedure, which is described in detail below. If desirable, the content of the unknown tubes can be estimated as an inverse proportionality (log-log function) to the known sample. Otherwise, the concentration of unknown iodide can be read from the chart shown in Figure 1.

Colorimetric Procedure. The colorimetric procedure is based upon the rate of

reduction of the yellow ceric ion to the colorless cerous ion. For this purpose one must (a) prepare suitable reagents, (b) determine the reduction rate of known standard solutions, and (c) estimate the unknown by comparison with the known rate of reduction.

The following reagents must be prepared:

Water. Distilled water is redistilled in an all-glass distillation apparatus containing about 20 grams of NaOH. The distillate is discarded until it becomes neutral to methyl red.

Other reagents. No attempt has been made to repurify the other reagents used, but the purest grade available has been selected.

Low-Nitrogen Sulfuric Acid (Mallinckrodt's C P) has been found virtually iodine free by the permanganate method (12). From this material a 7 N aqueous solution is made.

Standard solution of potassium iodide. This is a stock aqueous solution containing 261.6 milligrams of potassium iodide per liter.

Working standard. 1 cc of the stock solution is contained in 100 cc of solution, made up with distilled water.

Ceric ammonium sulfate. A reagent grade of ceric ammonium sulfate may be obtained from the G. F. Smith Company, Columbus, Ohio. The 0.05 M ceric solution used contains 29.73 grams per liter and is standardized occasionally against 0.1 N arsenious acid.

Arsenious acid 0.1 N (Mallinckrodt, A. R.) is prepared by dissolving 4.947 grams of As_2O_3 in 0.01 N H_2SO_4 , and making up to one liter volume.

Colorimetry. First of all one must determine the rate of reduction in the presence of known amounts of iodide. The procedure is as follows:

A convenient test tube, calibrated at 5 cc volume, is used. Into this tube is placed 106 milligrams of sodium carbonate (anhydrous), weighed within 1 milligram, or delivered quantitatively in the form of a 20 per cent aqueous solution. Half a cubic centimeter of water is added, and the desired aliquot of a standard iodide solution is added. Next 1.0 cc of 7 N sulfuric acid is added, or an appropriate amount corresponding to the acid in the companion unknown tube. Distilled water is then added carefully to the 5-cc line and the contents of the tube well mixed with a 3 cc pipette by drawing the solution into the pipette and expelling two or three times. Next, 3 cc of this solution is drawn up into the pipette, and the contents are placed in a Klett-Summerson colorimeter tube. To the 3 cc of solution is added 1.2 cc of distilled water and 0.2 cc of ceric ammonium sulfate solution (0.05 N). The contents of the colorimeter tube are well mixed by rocking the tube slightly or by tapping it, and the tube is held under a warm water tap at about 32 degrees centigrade. The time reaction will now begin on adding the arsenious acid solution (0.1 N).

With a stop watch in readiness, 0.5 cc of the arsenious acid solution is added, followed by a few drops of water to make the contents of the tube up to the 5-cc mark. At this time the stop watch is started at zero. The contents of the tube are stirred quickly with a small thermometer and held under the water tap to adjust the temperature close to 32 degrees centigrade. The outside of the tube is dried, and successive readings are made in the Klett-Summerson colorimeter.

at intervals of one minute until the residual substrate reading is approximately 130 on the dial. During the process the precise times at which the 160 mark and the 140 mark on the colorimeter was reached are noted. At the end of the

TABLE II

Typical protocols, showing catalytic effect of final sample (representing $\frac{1}{2}$ of the iodide indicated). Temperature, $32^{\circ}\text{C} \pm 1$

TIME minutes	PHARMACOLOGICAL RANGE (MICROGRAMS OF IODIDE $\times 100$)										
	0	10	20	30	40	50	60	70	80	90	100
0*	282?	283?	271?	?	276?	282?	307?	278?	258?	260?	260?
1	282	283	271	281	272	274	288	267	255	258	258
2	279	278	266	268	258	258	258	239	226	219	218
3	278	275	258	260	240	238	225	210	197	184	180
4	277	271	250	251	222	212	197	183	167	156	145
4.1											140
4.5										140	
5	275	269	243	238	206	195	170	158	140	129	118
5.7								140			
.6	274	267	238	227	193	175	147	134	117	105	95
6.4							140				
.7	272	266	230	216	177	158	128	114	102	87	80
7.95						140					
8	271	263	224	205	165	139	110	98	85	78	63
9	270	259	218	195	151	126	99	83	70	65	53
9.9					140						
10	270	259	212	183	138	115	87	70	59	54	43
11	270	256	206	174	131	102	75	59	48	47	37
12	269	253	200	164	122	87	64	50	40	38	31
13	269	250	194	154	112	80	55	43	34	33	27
14	269	248	189	145	104	74	48	36	29	28	24
14.5				140							
15	269	245	184	136	96	68	40	32	25	24	21
16	269	242	179	127	89	61	36	28	22	21	21
17	268	241	174	119	81	55	31	25	19	18	20
18	267	238	167	114	76	49	26	21	17	16	17
19	267	235	163	109	69	44	23	19	16	15	17
20	267	235	158	102	64	40	21	17	15	14	20
23.6			140								
63		140									

* The reading at zero time inevitably is taken late, and is therefore unreliable.

procedure the temperature in the solution is taken again, and the average temperature over the entire course of the reaction is estimated.

The success of this method depends upon the colorimeter's being so well insulated that it itself acts as a thermostatic air bath. With a little ingenuity this arrangement may be achieved, and the great convenience of avoiding a separate thermostatic apparatus compensates for the small loss in accuracy which

is inevitable For greater accuracy a thermostat water-bath may be employed, and the extent of reduction measured at intervals of five minutes

TABLE III

Calculation of iodide based on time required (at $52^{\circ}\text{C} \pm 1$) to reach a given residual substrate concentration

KNOWN IODIDE $\mu\text{g} \times 100^*$	TIME IN MINUTES REQUIRED TO REDUCE RESIDUAL SUBSTRATE READING AT		PRODUCT OF TIME \times IODIDE AT		IODIDE CALCULATED AT	
	160/252	140/252	160	140	160	140
20	19.5		39.0		17	
30	12.5	14.5	37.5	43.5	27	28
40	8.2	9.9	32.8	39.6	41	40
50	6.8	8.0	34.0	40.0	Taken at 50 as standard	
60	5.4	6.4	32.4	38.4	63	63
70	4.8	5.7	33.6	39.9	71	70
80	4.2	5.0	33.6	40.0	81	80
90	3.8	4.5	34.2	40.5	90	89
100	3.5	4.1	35.0	41.0	97	95
At 160 $\frac{6.8 \times 50}{T} = \frac{340}{T}$			At 140 $\frac{5 \times 80}{T} = \frac{400}{T}$			

* Although the iodide samples represent amounts expected in 1 cc. of serum, they are presented in terms of concentration per 100 cc.

TABLE IV

Calculation of iodide based on residual substrate concentration at certain time intervals

AMOUNT OF IODIDE μg	READING AT END OF FOUR (4) MINUTES	READING AT END OF FIVE (5) MINUTES	READING AT END OF TEN (10) MINUTES
0	277	275	270
1	271	269	259
2	250	243	212
3	251	238	183
4	222	206	138
5	212	195	115
6	197	170	87
7	183	158	70
8	167	140	57
9	156	129	54
10	145	118	43

Computation of standard curves In Tables II, III and IV are presented characteristic data obtained with known amounts of iodide. It will be observed that these data may be used in one of two ways. First, it will be noted that under certain fortuitous combinations of conditions the time required to reach a certain

reading, e.g., 140, is inversely proportional to the amount of iodide present when plotted on log-log paper. Such calculations are illustrated in Table II, both for the reading 160 and the reading 140. Secondly, estimation of the iodide present may be based on the residual substrate concentration at certain definite time intervals. This method is illustrated by Table III.

It is convenient to have these data plotted in the form of graphs as illustrated in Figure 1, which simply presents in graphic form the data of Table II. It will be noted from Figure 1 that the time intervals at which a certain colorimeter reading is reached is nearly a straight line when plotted on log-log paper against the corresponding samples of iodide.

DISCUSSION. It should be emphasized that a number of variables influence the speed of this reaction. Among these, the chief are 1) iodide concentration, 2) temperature, 3) concentration of electrolyte, and 4) concentration of hydrogen ions and other specific ions, notably osmium (6). The method, therefore, involves a high degree of empiricism, and it is essential that each worker determine his own nomogram (11), according to the circumstances under which the catalytic effect is measured. Because the rate of reaction is influenced by the total electrolyte content of the system, the total sodium in the solution must be controlled accurately.

The general method under discussion has been tried and discarded in several laboratories because it was assumed that the rate of reduction would be constant in all laboratories and under all conditions, without due regard to the several variables involved. Absolute control of these several variables is essential. When the proper precautions are observed, however, the method is simple and rapid in comparison with micromethods now in use.

Because of the empirical nature of this method, it is essential that each investigator determine his own charts of standard values for known amounts of iodide. The chief difficulty which may arise in the colorimetric procedure is through accidental contamination with mercury. It was demonstrated by Sandell and Kolthoff (6) that mercuric ion "poisoned" the catalytic effect of the iodide by forming mercuric iodide which is insoluble. If this contamination should occur, as suspected from lack of progress in the reaction, it may be "tested for" as follows. A standard amount of known iodide is added to such a reaction mixture immediately after the usual colorimetric procedure. Then an appropriate amount of ceric solution is quickly added, and the course of the reaction is followed as usual. A slight correction must be made at the end for the undue dilution of the reaction mixture. From such an experiment, however, it will be seen quickly whether the environment is favorable to the catalytic reduction of the ceric ion.

In the course of time, both the working standard solution and the arsenious acid become weaker in their effects. The precise reasons for this are not yet clear, but the difficulty can be met readily by renewing the solutions. In particular, the weakening of the arsenious-acid reductant tends to slow the course of the reaction. This result will be detected if occasional known solutions of iodide are measured, as controls.

Certain modifications may be adopted by the individual worker. For example, instead of reading every minute it may suffice to read every five minutes, or to take the reading at the end of a known period.

In some cases it will be desirable to separate the protein-bound iodine from the total iodine. This procedure is not discussed in the present communication, because it has been described at length elsewhere (12, 13, 14).

SUMMARY

Although the measurement of iodine concentration in the blood plasma is of considerable interest in connection with the use of iodine-containing drugs, the best methods now available require nearly 40 cc. of blood for duplicate analyses of blood at physiological concentrations. This fact limits the use of such methods in man, and prohibits them in small animals. The present communication describes a micro-analytical technique which will yield duplicate analyses from 2 cc. of blood plasma or serum. The method involves the catalytic effect of iodide upon the reduction of ceric ion. The technique is colorimetric and is adaptable to standard photoelectric colorimeters such as are available in many laboratories at the present time.

REFERENCES

1. SALTER, W. T., *The Endocrine Function of Iodine*, Harvard University Press, Cambridge, 1940.
2. SALTER, W. T., BASSETT, A. M., AND SAFFINGTON, T. S., Protein bound iodine in blood. VI. Its relation to thyroid function in 100 clinical cases. *Am J Med Sci*, **202**, 527, 1941.
3. RIGGS, D. S., GILDEA, E. F., MAN, E. B., AND PETERS, J. P., Blood iodine in patients with thyroid disease, *J Clin Invest*, **20**, 345, 1941.
4. SALTER, W. T., SAFFINGTON, T. S., AND MCKAY, E. A., Iodine in Blood and Thyroid. VIII. An Analytical Procedure for Use with Small Samples. Physiological Range of Concentrations. To be published.
5. CHANEY, A. L., Improvements in determination of iodine in blood. *Industrial and Engineering Chemistry, Analytical Edition*, **12**, 179, 1940.
6. SANDELL, E. B., AND KOLTHOFF, I. M., Microdetermination of iodine by catalytic method. *Microchimica Acta* **1**, 9, 1937.
7. SUMMERSON, W. H., A simplified test tube photoelectric colorimeter, and the use of the photoelectric colorimeter in colorimetric analysis. *J Biol Chem*, **130**, 149, 1939.
8. KENDALL, E. C., The determination of iodine in connection with studies in thyroid activity. *J Biol Chem*, **19**, 251, 1914.
9. PERKIN, H. J., determination of iodine in blood. *Biochem J*, **27**, 1078, 1933.
10. GNOOK, B., *Midrogesamtjodbestimmung und Jodbestimmung in organischen SAUREN*. *Biochem Ztschr*, **175**, 455, 1925.
11. DAVIS, D. S., *Empirical Equations and Nomography*. McGraw Hill Book Co., Inc., New York and London, 1943.
12. BASSETT, A. M., COONS, A. H., AND SALTER, W. T., Protein bound iodine in blood. V. Naturally occurring iodine fractions and their chemical behavior. *Am J Med Sci*, **202**, 516, 1941.
13. MAN, E. B., GILDEA, E. F., AND SMIRNOW, A. L., Serum iodine fractions. *Federation Proceedings*, **1**, Pt. II, 123, 1942.
14. MAN, E. B., SMIRNOW, A. E., GILDEA, E. F., AND PETERS, J. P., Serum iodine fractions in hyperthyroidism. *J Clin Invest*, **21**, 773, 1912.
15. RIGGS, D. S., AND MAN, E. B., A permanganate acid ashing micromethod for iodine determinations. I. Values in blood of normal subjects. *J Biol Chem*, **134**, 193, 1940.

CHANGES IN ACTIVITY OF PULMONARY RECEPTORS IN ANAESTHESIA AND THEIR INFLUENCE ON RESPIRATORY BEHAVIOUR

D. WHITTERIDGE AND E. BÜLBRING

From The Departments of Physiology and Pharmacology, Oxford

Received for publication May 8, 1944

There is a number of conditions in which rapid and shallow breathing occurs in spite of the absence of any recognised change in the chemical stimulus to the respiratory centre. These include experimental starch embolism (Dunn, 1); (Binger, Brow and Branch, 2), perhaps cardiac dyspnoea (Christie, 3), and blast injury to the lungs (Krohn, Whitteridge and Zuckerman, 4). During some work on these conditions, we encountered complaints of respiratory disturbance during anaesthesia with trichlorethylene. This anaesthetic produces rapid and shallow breathing which is very readily reversible. We have therefore investigated the effects of trichlorethylene on the vagal afferent systems, and have compared them with those of other anaesthetics which do not usually cause conspicuous respiratory changes.

METHODS

For the study of the activity of vagal endings in the lungs we have used cats, decapitated under ether and left for 1-2 hours to allow the anaesthetic to blow off. 2 mg. atropine was injected before to prevent bronchial secretion and reflex bronchial constriction. Their lungs were inflated by a small all-metal pump of variable speed and stroke volume with an electromagnetic expiratory valve, which opened at the end of inflation. The arrangement is similar to that of a Starling Ideal Pump. The resistance to inflation was measured optically by a calibrated membrane manometer connected to the side tube of the tracheal cannula.

Single fibre preparations were obtained by cutting down the vagus with sharp needles and mounting on moisture-resisting electrodes (5).

Impulses were recorded with a resistance-capacity coupled amplifier of conventional design and a cathode ray tube, and simultaneously followed with a loudspeaker. The animals' head and chest lay in a moist chamber. In a few experiments the pressure in the right ventricle has been recorded by inserting into it a needle which was connected by a lead tubing to a membrane manometer similar to that described by Hamilton, Brewer and Brotman (6). Possible changes in the resting level of air in the chest were minimised by allowing as long as possible for expiration, and checked by including a 10 l. reservoir bottle in a closed circuit with the pump and the cat, and recording the pressure changes in the bottle optically from a third membrane manometer. No CO₂ absorbent was included, and the closed circuit was therefore used for short periods only.

In a few experiments, curarised decapitate and decerebrate animals were

used in order to exclude any active contraction of respiratory muscles. There were no differences between the behaviour of these animals and that of decapitate animals without curare.

For the investigation of changes in the volume of air in the chest decerebrate and anaesthetised animals were enclosed in an air tight respiration chamber of about 15 l capacity with external connection for the tracheal tube. The air displaced from the chamber was recorded by a small Krogh spirometer. In some experiments, impulses in vagal single fibre preparations were recorded simultaneously. Small doses of nembutal were sometimes given to these decerebrate cats in order to diminish their hyperexcitability.

In the course of some perfusion experiments on cats' lungs *in situ*, to be published elsewhere with a full description of the technique, we made a few observations of the action of anaesthetics for control purposes.

In experiments on rabbits a sample of diaphragmatic activity was obtained by recording the contractions of a diaphragmatic strip. The technique described by Head (7) was closely followed, with the exception that we sometimes split the diaphragm in the mid line and used only one of the diaphragm strips. For cooling the vagi we used a silver plated tube bent in the shape of a W and slipped under both nerves. This was cooled by brine and its temperature recorded by a thermocouple on the outflow.

The anaesthetics were administered by means of Oxford vaporisers specially adapted and calibrated in volume percentage for use with animals by Dr H Epstein of the Nuffield Department of Anaesthetics. Mixtures of cyclopropane and of nitrous oxide with oxygen (containing 5% CO₂) were made up in Douglas bags. Our source of trichlorethylene was Trilene made by ICI (Pharmaceutical), and we used chloroform and ether from different manufacturers. For divinylether, we used Vinesthene (May and Baker, Ltd) containing 96.5% divinylether and 3.5% absolute alcohol.

RESULTS

1 Experiments on decapitated cats

In by far the largest number of vagal single fibre preparations the impulses have a respiratory rhythm and increase in frequency with expansion of the lungs. Such 'stretch' endings produce an inhibition of inspiration, (8). The exact situation of these endings is still uncertain, histologically, appropriate sensory endings have been found from the respiratory bronchioles to the alveoli by Elftmann (9). In decapitated cats the majority of fibers from stretch receptors are inactive during expiration. During inspiration they become active and the frequency of discharge increases with expansion of the lungs. With an inflation of 100 cc the peak frequency of discharge in different fibres varies from 50-150 impulses per sec. Adrian (8) has shown that with maintained inflations the final steady frequency reached depends only on the volume of air in the chest, not on the rate of inflation. With rapid inflation there is at first a high rate of discharge which falls to a steady level. In order to avoid this initial rapid discharge we used a pump working at 15 revolutions per minute which took 2

seconds to inflate the lungs with 40–100 cc. of air. With this arrangement the highest frequency reached was well maintained and never fell more than 10% during the remainder of the period of inflation. With larger inflations this peak frequency is directly proportional to the pump stroke (8), but there may be some deviation below 70 cc. Frequency-volume curves are shown in fig. 1 and a record of impulses in fig. 2. In all our preparations, endings, which were stimulated during inflation, were of the slowly adapting type and care was taken to avoid over-inflation at any stage. As sufficient time was allowed for the lungs to empty themselves completely between each pump stroke, the peak frequency of dis-

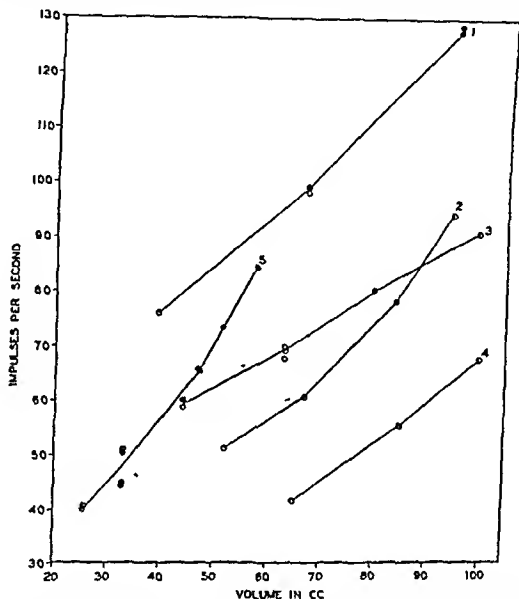


FIG. 1. RELATION BETWEEN THE VOLUME OF AIR IN THE CHEST (ABSCISSA) AND THE FREQUENCY OF DISCHARGE FROM STRETCH ENDINGS (ORDINATE)

1, 2, 3, 4: decapitate preparations inflated by pump; 5: decerebrate cat, volume measured in respiration chamber.

charge with constant inflation remained the same within $\pm 5\%$ as long as the fibre survived which varied from $\frac{1}{2}$ –3 hours.

When trichlorethylene was added to the inspired air, there was a steady increase in the peak frequency of discharge in spite of the constant output of air from the pump. With the lowest concentrations ($\frac{1}{2}$ –1%) increases of frequency from 30–50% were seen. With 1–2% trichlorethylene this increase in frequency continued until it reached 50%–140% above the initial value, where it was maintained. With higher concentrations there was an increase in the rate of adaptation within a few seconds, so that the peak frequency was reached earlier and earlier during inspiration until impulses ceased half-way through the pump stroke; this was followed by complete failure of the ending (see fig. 3). During

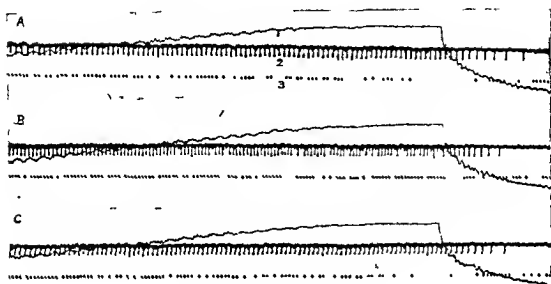


FIG 2 DECAPITATE CAT

Records of (1) tracheal pressure, (2) action potentials from a single stretch ending (3) time in $\frac{1}{16}$ and $\frac{1}{8}$ secs Inflation with constant volume A before, B during, C after exposure to 2% trichlorethylene

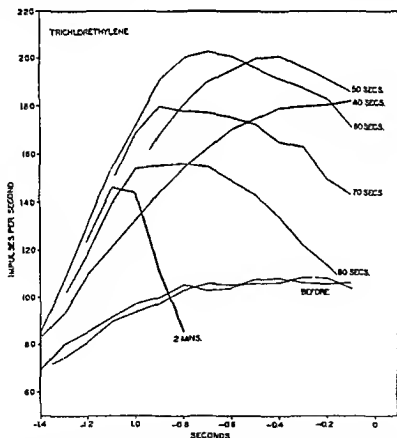


FIG 3 THE EFFECT OF 3-4% TRICHLORETHYLENE ON THE RESPONSE OF A STRETCH ENDING DURING SINGLE PUMP STROKES

Decapitate cat, inflated with constant volume. Ordinate = frequency of impulses per sec, abscissa = time in $\frac{1}{16}$ secs before the end of inflation at 0

recovery, which occurred very rapidly after withdrawal of the trichlorethylene, there was a further period of increased sensitivity during which the frequency rose to 50–60% above normal and slowly returned to its initial value. In fig. 4a the initial effect of giving trichlorethylene was a rise in frequency from 103 to 198 per sec., then a drop to 49 per sec., and on withdrawal a rise to 178 per sec. After 9 minutes' exposure to the drug about 30 minutes were needed for the frequency to return to normal; in another experiment after 2 minutes exposure the frequency returned to normal after 20 minutes. The increase in frequency

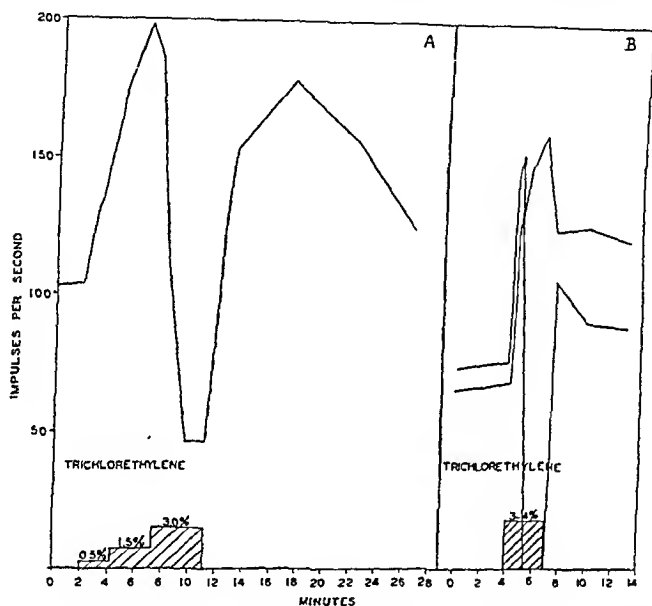


FIG. 4. THE EFFECT OF TRICHCLORETHYLENE ON THE PEAK FREQUENCY OF DISCHARGE OF A STRETCH ENDING

Ordinates = impulses per sec., abscissa = time in minutes. A: Exposure to 0.5, 1.5 and 3% trichlorethylene. B: Simultaneous record of two endings, both of which are stimulated, but one only is paralysed by 3–4% trichlorethylene.

of discharge with trichlorethylene was invariably seen. When impulses from two or three fibers were recorded simultaneously some difference in their sensitivity to the anaesthetic was usually seen (see fig. 4b); all fibres were paralysed with concentrations above 3.5% trichlorethylene.

Other volatile anaesthetics tested, i.e. chloroform, ether, divinylether, ethylchloride, cyclopropane and nitrous oxide had the same type of effect as trichlorethylene on stretch endings.

With chloroform the most conspicuous feature was the rapidity of its action. With low concentrations (1%) there was a very rapid increase in frequency which was however not maintained; e.g., in the experiment shown in fig. 5a the fre-

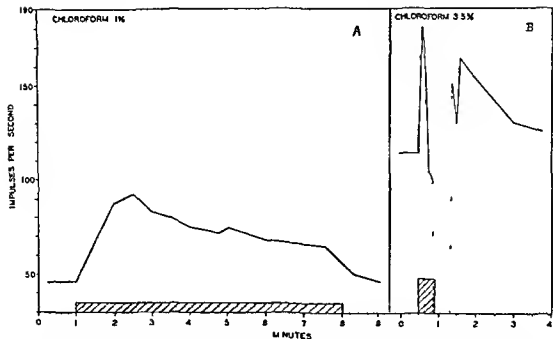


FIG 5 THE EFFECT OF (A) 1% CHLOROFORM AND (B) 3.5% CHLOROFORM ON THE PEAK FREQUENCY OF DISCHARGE FROM A STRETCH ENDING
Ordinate = impulses per sec, abscissa = time in min

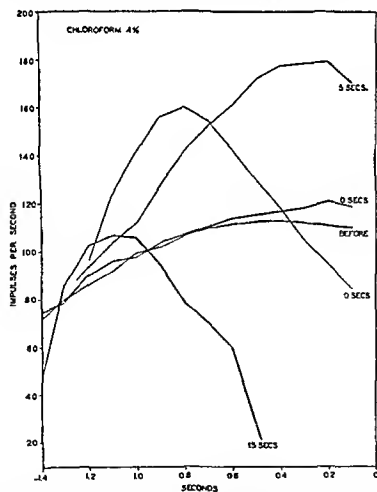


FIG 6 THE EFFECT OF CHLOROFORM (APPR 4%) ON THE RESPONSE OF A STRETCH FADING DURING EACH SUCCESSIVE PUMP STROKE
Ordinate = impulses per sec, abscissa = time in $\frac{1}{10}$ sec before the end of inflation at 0

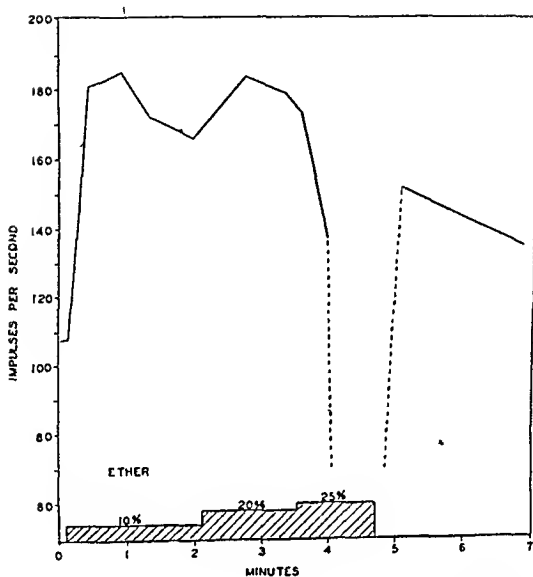


FIG. 7. THE EFFECT OF ETHER ON THE PEAK FREQUENCY OF DISCHARGE FROM A STRETCH ENDING

The ether concentration was increased from 10% to 20% and finally to 25%. Ordinate = impulses per sec., abscissa = time in min.

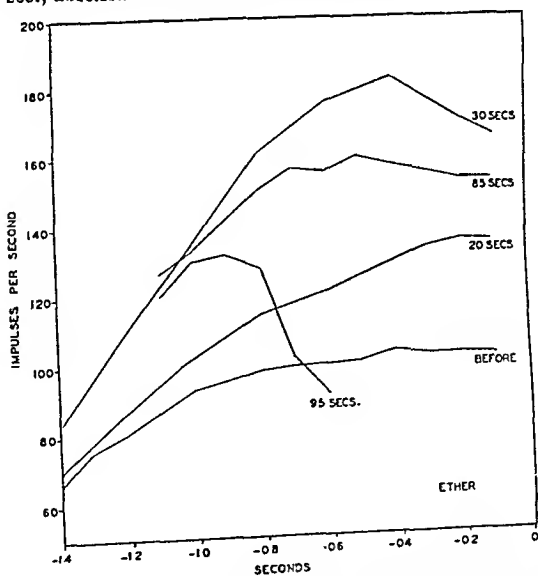


FIG. 8. THE EFFECT OF ETHER ON THE RESPONSE OF A STRETCH ENDING DURING SINGLE PUMP STROKES

Ordinate = impulses per sec.; abscissa = time in $\frac{1}{10}$ sec. before the end of inflation at 0

quency rose from 46 to 87 impulses per sec in 45 seconds, during the next 45 seconds to 91, but then during the next five minutes of exposure it fell gradually to 65 per sec. With higher concentrations of chloroform (3-4%) the increased frequency was evident only for 10 seconds and the ending had failed completely at the end of 20 seconds (see fig 5b). The failure occurring earlier and earlier during the pump stroke can be seen in fig 6. After stopping the chloroform, recovery was equally rapid and for a short period the ending was again hyperexcitable. An effect very similar to that of chloroform has been seen with carbon tetrachloride but has not been studied in detail.

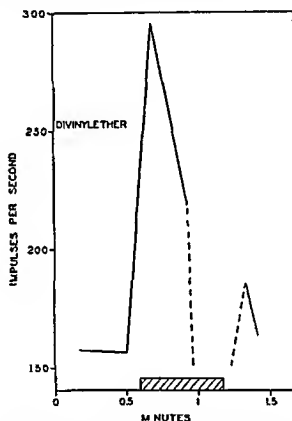


FIG 9 THE EFFECT OF 4% DIVINYLETHER ON THE PEAK FREQUENCY OF DISCHARGE FROM A STRETCH ENDING

Ordinate = impulses per sec, abscissa = time in min

With ethyl ether the onset of changes in frequency was not quite as rapid as with chloroform. The increased discharge caused by low concentrations amounted to 30-100% in various preparations but was not well maintained. High concentrations produced early failure. In fig 7 an experiment is shown in which 10% ether produced a rise in frequency from 108 to 184 per sec which was not maintained. When 20% ether was substituted there was a second rise to 183 per sec, but when 25% ether was administered the frequency of discharge decreased and after a few seconds the ending failed completely. Rapid recovery with hyperexcitability was seen on withdrawal. Fig 8 shows the changes in discharge during single pump strokes in a similar experiment.

With as little as 4% divinylether increased excitability followed by failure of a

stretch ending has been seen (fig. 9). A few observations on the action of ethylechloride suggested that this was the most potent substance tried.

Cyclopropane and nitrous oxide produced a rise in frequency (50–100%) which quickly reached a steady level, and during brief inflation with 100% of either, no sign of failure was seen. Fig. 10 shows the rapid rise in excitability to a steady level during exposure to 50% cyclopropane and 80% nitrous oxide and the equally rapid fall to the original level. The steadiness of this action of cyclo-

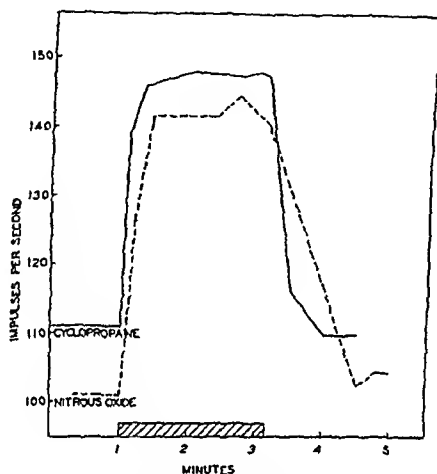


FIG. 10. THE EFFECT OF 50% CYCLOPROPANE (CONTINUOUS LINE) AND OF 80% NITROUS OXIDE (BROKEN LINE) ON THE PEAK FREQUENCY OF DISCHARGE FROM A STRETCH ENDING

Ordinate = impulses per sec.; abscissa = time in min.

propane was utilised to investigate the change in peak frequency with different stroke volumes, as is shown in the following table.

VOLUME OF AIR cc.	FREQUENCY OF DISCHARGE PER SEC.		INCREASE %
	Normal	During exposure to 50% cyclopropane	
54	42.5	93.5	120
67	52.5	120.0	128
95	106.0	255.0	141

Over the range of volumes studied the percentage increase in the peak frequencies remained approximately the same.

When recording from a single vagal ending in the perfused lung we have observed the same effects of trichlorethylene as those described in the whole animal; they cannot therefore be due to an action of the anaesthetic on the heart. The effects were also independent of the route of administration of the anaesthetic,

as acceleration and paralysis occurred in the same way after intravenous injection

No method of producing asphyxia of the endings has caused comparable changes in sensitivity to stretch. A few minutes' exposure to nitrogen has no effect on the ending, confirming (8). Injection of 100 mg potassium cyanide was followed by a fall in frequency from 80 to 60 per sec without preceding acceleration; later the frequency rose to 103 per sec and the ending became insensitive to stretch for some seconds before complete failure. This may possibly correspond to the rapid firing seen by Matthews (10) in asphyxiated stretch endings of striated muscle.

With intravenous injection of nembutal a very transient fall in frequency of impulses without preceding stimulation has been recorded. With chloralose injected intravenously in doses of 100 mg per kg there was no significant change in frequency.

Either no change or a slight decrease in resistance of the lungs to inflation (not more than 5%) has been seen during the period of increased activity of the stretch endings. When the anaesthetic concentration was increased to the point of cardiac failure, indicated by a decrease in right ventricular pressure, there was sometimes a considerable increase in the resistance of the lungs to inflation.

Considering our results obtained so far it seemed that in general the initial effects of these volatile anaesthetics in increasing the sensitivity of the stretch endings were remarkably alike. The difference between them lay in the rate at which this increased sensitivity decayed during continued exposure to the same concentration of the anaesthetic. Thus the increase produced by nitrous oxide and cyclopropane was maintained as long as the observation was continued, i.e. 5 minutes. On the other hand during continued exposure to 1% chloroform the increase in the peak frequency was halved in 5 minutes.

2 Experiments on cats breathing spontaneously

In all experiments on the administration of anaesthetics to spinal animals, we had practically no indication of the depth of anaesthesia reached. In a small series of experiments in which we recorded the pressure in the right ventricle, there was some evidence of the onset of a direct toxic effect on the heart. We wished, however, to find out if failure of the endings preceded respiratory arrest, and if the sensitisation of the endings observed in spinal animals had any significant effect on the respiration as a whole. Accordingly, we made observations on the effect of these anaesthetics on the respiration of decerebrate cats. In order to obtain quantitative records of the respiratory rate, depth and functional residual air, the animals were enclosed in a respiration chamber, with the tracheal tube connected externally. Fig. 11 shows the effects of ether and trichlorethylene on the same animal, and fig. 12 that of cyclopropane on another animal. In each case there was an initial decrease in the depth of respiration, which could be due to the sensitisation of the stretch endings. In these experiments, however, the respiratory pattern might have been modified by effects of the anaesthetics

on extra-pulmonary receptors and on the respiratory centre itself. We therefore did experiments on doubly vagotomised animals. With ether there was an

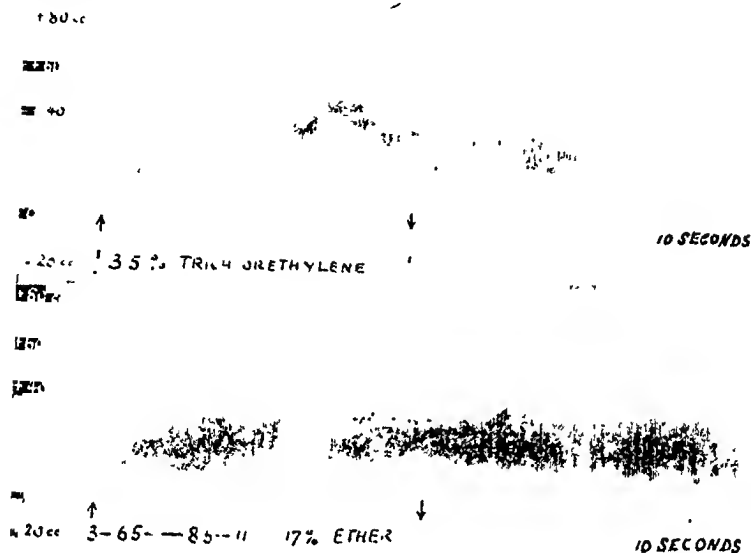


FIG. 11. DECEREBRATE CAT IN RESPIRATION CHAMBER

Inspiration upwards. A: Record of respiration during exposure to 3.5% trichlorethylene; B: 30 min. later, during exposure to increasing concentrations of ether. Note the onset of change in respiratory pattern immediately after a deep breath.

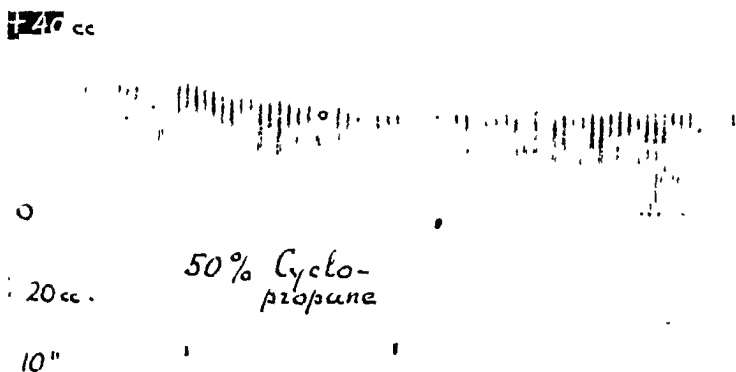


FIG. 12. DECEREBRATE CAT IN RESPIRATION CHAMBER

Inspiration upwards. Record of respiration during exposure to 50% cyclopropane

increase in rate from 13 to 175 per min. as compared with an increase from 36 to 64 per min. produced by the same concentration of ether before vagotomy. When the vagi were cooled to 1°C., we have had no evidence in cats of any

stimulation of the respiratory centre by trichlorethylene, though this sometimes occurred in rabbits after several minutes. According to Adrian (11) chloroform increases the respiratory rate considerably in rabbits after double vagotomy. Heinbecker and Bartley also found some acceleration with ether in vagotomised cats, and in view of its stimulating action on the electrical responses of other

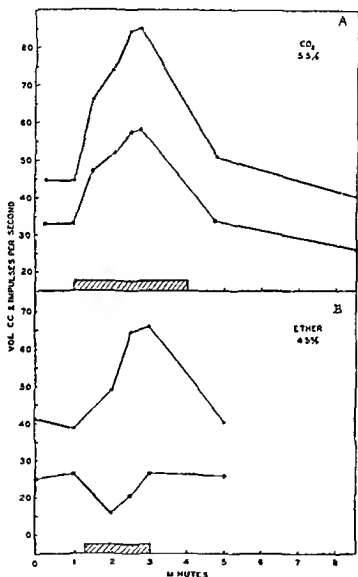


FIG. 13. DECREASED CAT IN RESPIRATION CHAMBER

The time course of changes in frequency of discharge from a stretch ending (dots) and the volume of air in the chest (circles) at the end of inspiration. A during administration of 5.5% CO₂. B during administration of 4.5% ether. Ordinate = impulses per sec and lung volume in cc. abscissa = time in min.

parts of the central nervous system this is hardly surprising. Possibly bigger effects might have been seen on the respiratory centre in the absence of basal doses of nembutal which we used.

In order to identify effects on the stretch receptors themselves we recorded the activity in single fibres simultaneously with the changes in lung volume. Fig. 13a shows the effect of a chemical stimulus on the respiratory centre, in this case

the inhalation of 5.5% CO_2 . The changes in lung volume and in peak frequency of discharge are closely parallel. On the other hand, in fig. 13b and fig. 14 there is an increased frequency of discharge in the stretch fibre at the same time as a decrease in lung volume, following exposure to ether and to trichlorethylene. In these experiments, in which one or both vagi were intact, the frequency of discharge of the stretch ending, probably a fair sample of the stretch endings as a whole, largely determined the depth of the respiration. Fig. 14 shows

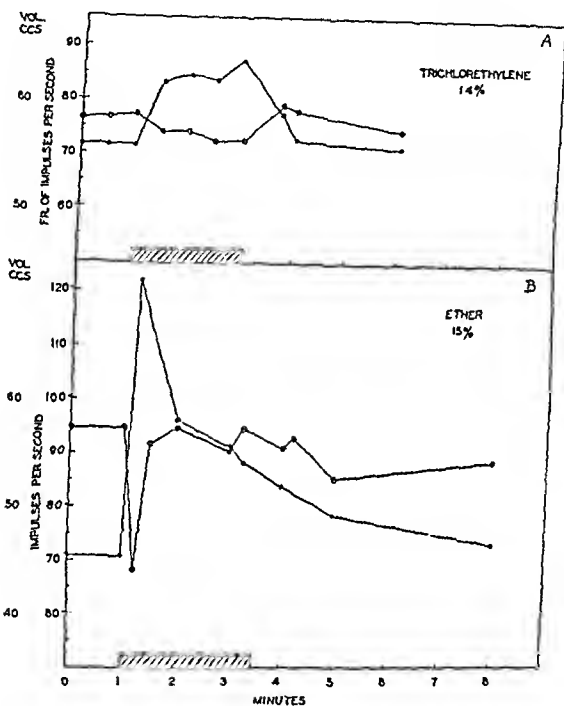


FIG. 14. DECEREBRATE CAT IN RESPIRATION CHAMBER

The sequence of changes in frequency of discharge from a stretch ending (dots), and the volume of air in the chest (circles) at the end of inspiration. A: during exposure to 1.4% trichlorethylene, B: during exposure to 15% ether. Ordinate = impulses per sec. and lung-volume in cc.; abscissa = time in min.

clearly that, just as in the spinal preparations, the sensitisation of the stretch endings by ether is often transitory, whereas that due to trichlorethylene develops more slowly and persists throughout exposure.

With 15% ether the early sign of failure, cessation of the discharge before the end of inspiration, frequently occurred, and complete failure of the ending was repeatedly seen well before respiratory arrest. This may account for the observation of McDowall (13) that section of the vagi under deep ether anaesthesia has no effect on the respiration. During recovery from deep ether anaes-

thetia, there was usually a period of increased excitability of the ending similar to that seen in the spinal cat

With high concentrations of trichlorethylene signs of impending respiratory arrest have been observed in a few experiments before failure of a group of stretch endings. In this respect trichlorethylene resembles chloroform which produces respiratory arrest at about the same time as failure of the stretch endings (8).

Although there does seem to be a close relation between the sensitivity of the stretch endings and the depth of respiration, the respiratory rate and the expiratory level of the chest seem to vary independently (figs 11 and 12). The increase in functional residual air has been most marked with trichlorethylene and has amounted to 6.38 cc. With concentrations of 3% the extent of the increase varied with the rate of induction. By recording diaphragm action potentials from a needle electrode a discharge which continued throughout expiration was observed at the peak of the increase in functional residual air. In view of the difficulties in recording slight changes in muscle tone with needle electrodes sampling only those motor units near the needle, the assumption may be justified that the observed increase in functional residual air is due to a maintained tone in the diaphragm. The only alternative explanation namely local bronchoconstriction, is ruled out by the experiments using artificial respiration in which there was no increase in the resistance to inflation. Possible reflex bronchoconstriction was eliminated by atropine.

Chloroform seemed to have little effect on either respiratory rate or expiratory level of the lungs, but trichlorethylene increased both. Ether often caused an increase followed by a decrease in both, while cyclopropane had little effect on the expiratory level but caused considerable slowing of the respiration. As it seemed impossible to ascribe these different effects to an action on the stretch endings on which all these anaesthetics have qualitatively similar effects, we investigated the possibility that they might modify the behaviour of other afferent endings.

3 Experiments on the diaphragm slip preparation in rabbits

As vagotomy almost abolished the effects of trichlorethylene on the respiration it seemed that stimulation of extrapulmonary receptors was unlikely to be of great importance. We therefore turned our attention to pulmonary receptors other than the stretch endings. The existence of separate endings stimulated by deflation has been maintained by Head (7) and supported by the direct observations by Adrian (8) and the study of partial vagal block by Hammond and Wilson (14). We have recorded the activity and the tone of the diaphragm using Head's method. For anatomical reasons this can only be done in the rabbit.

With intact vagi as Head has shown inflation of the lungs leads to abolition of the resting tone of the diaphragm and slowing or cessation of the respiratory movements. On the other hand suction of air from the chest leads to an increase of diaphragm tone which may be accompanied by an increase or decrease of respiratory rate. According to Creed and Hertz (15) a suction pressure of

less than 2 cm. Hg causes a quickening, above 2 cm. Hg a slowing. In our experiments the suction of 1.3–1.6 cm. Hg was applied for 15" and the response was always an acceleration. In the cat, deflation of the chest produced either no change or a slowing of the respiratory rate but was accompanied by an immense increase in diaphragm tone; thus a multi-fibre record from needle electrodes in the diaphragm showed a great continuous increase in activity.

When the vagi of a rabbit were cooled to 3–5°C., Head's paradoxical response to inflation appeared: this consists of a the replacement of the normal relaxation of the diaphragm by a contraction followed by a number of inspirations. The

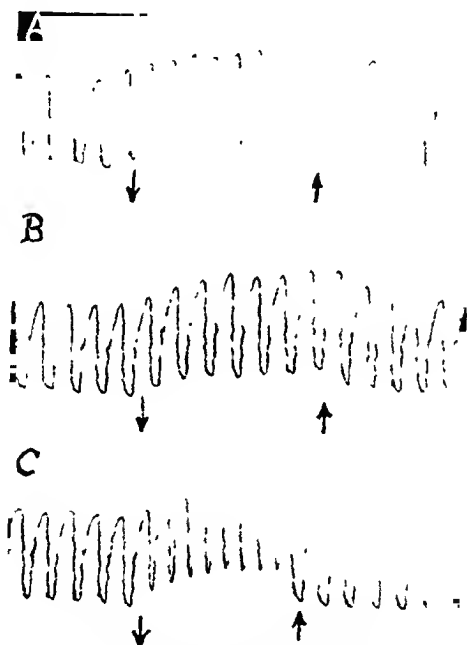


FIG. 15. RABBIT, CHLORALOSE

Record of diaphragm slip preparation. Response to 15" suction of air from the trachea. Both vagi were cooled in A to 4°C, in B to 1°C, in C to 4°C.

acceleration in response to suction however remained (cf. Hammouda and Wilson (14)). After cooling the vagi to 1°C. all reflex effects of deflation on diaphragm tone and on respiratory rate were abolished as is shown in fig. 15. The slight increase in baseline during suction while the vagi were cooled to 1°C. was attributed to an artefact. These results agree well with Hammouda and Wilson's interpretation that at a temperature of 3–5°C. the impulses from stretch endings are blocked. As Partridge (16) has shown, at this temperature impulses producing inspiratory effects still reach the centre.

With intact vagi the immediate effect of giving ether by tracheal tube to a rab-

bit was to produce a series of violent inspiratory efforts followed by a prolongation of the inspiratory phase and a shortening of the expiratory pause (see fig 16a). This subsided within a few minutes, and after stopping the ether it was not possible to obtain the same effect again for about 1-1½ hours (see fig 16b). A similar stimulation can be seen to a lesser degree with trichlorethylene, chloroform and divinylether. These observations are strongly suggestive of stimulation of the bronchial mucous membrane producing efforts to cough.

Some of the mechanisms involved in the immediate effect of anaesthetics can be separated out by cooling the vagi. At a temperature of 4°C deflation reflexes are still obtainable whereas stretch afferents are completely blocked. A conspicuous change in rate and depth of respiration was always seen when the

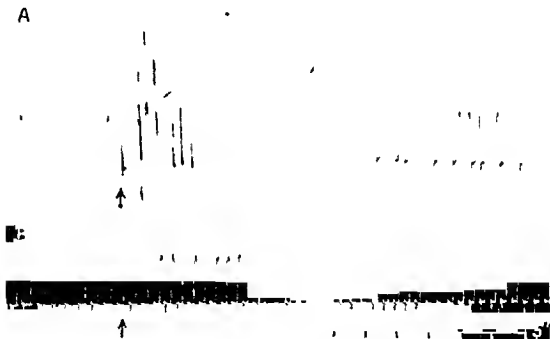


FIG 16 RABBIT CHLORALOSE

Diaphragm slip preparation. Vagi intact. A Immediate effect of 8% ether which was stopped after 2 min. and administered once more (B) after an interval of 13 minutes.

temperature in the cooling tube fell from 7° to 4°C. When the animal was exposed to ether while the vagi were kept at 4°C there was still an increase in diaphragmatic tone and in the amplitude of the contractions of the slip with comparatively little change in rate. This increased tone subsided during continued administration of ether within a minute or two, whereas with trichlorethylene a small but gradually increasing tone was observed. This is shown in fig 17.

The sequence of events in an animal under ether and with the vagi cooled to 4°C was as follows. During the first minute or two after induction, suction of air from the chest produced an increase in diaphragm tone and an acceleration of respiration which was sometimes even greater than before the anaesthetic. On continuing the same concentration of ether, deflation of the chest produced

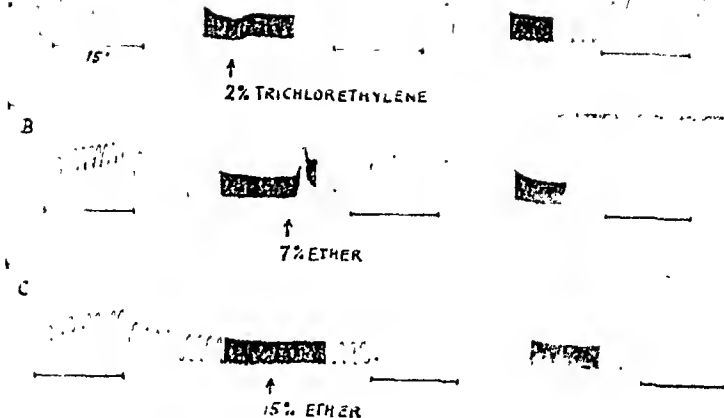


FIG. 17. RABBIT, CHLORALOSE, DIAPHRAGM SLIP PREPARATION

Vagi cooled to 4°C throughout. The immediate effects of trichlorethylene (A) and ether (B) on diaphragm tone are shown with a slow drum. The acceleration due to deflation (shown on a faster drum) persists during continued administration of trichlorethylene (A); but with ether this acceleration is increased at first (B), and is later replaced by slowing (C).

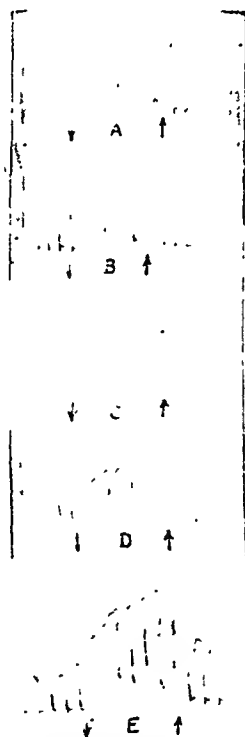


FIG. 18. RABBIT, CHLORALOSE, DIAPHRAGM SLIP PREPARATION

The effect of 15" suction is shown, A: with intact vagi before; B; C; D: during administration of 8% ether, B: vagi intact, C: vagi cooled to 4.2°C, D: vagi cooled to 1°C. E: 1 hour 20 min. after stopping the ether, vagi cooled to 5°C.

less and less acceleration and finally the first breath or two during suction was considerably prolonged (see figs. 17c and 18). The increased diaphragm tone was, however, always observed even if the ether concentration was raised to 15%. During exposure to trichlorethylene, suction of air from the chest while the vagi were cooled to 4°C. produced either the same effect as before or a slightly increased acceleration as well as an increase in tone. This effect persisted throughout the exposure (see fig. 17n).

It appears then that there is one mechanism which is stimulated by ether as well as trichlorethylene and which survives cooling to 4°C. This mechanism is, however abolished by more prolonged exposure even to low concentrations of ether whereas it is apparently unaffected by trichlorethylene. In addition there seems to be a further mechanism which also survives cooling and also survives 15% ether; this mechanism must be responsible for the persistence of the increase in tone and the slowing of respiration during suction.

DISCUSSION

Previous investigations on the effect of drugs on stretch endings in the lungs are few, possibly because various workers have stated that no significant effect occurs before paralysis. Thus Head (7) said that ether and chloroform paralysed vagal endings, in spite of the fact that Kandaraczi (17) observed that when chloroform was administered by tracheal tube, 1½-2% produced rapid and shallow breathing in a cat and that this was abolished by cutting both vagi. He attributed the shallow breathing to irritation of the vagal nerve endings in the lungs. Keller and Loeser (18) failed to observe any stimulating effect with ether. This may be attributed partly to their failure to record the volume changes in the chest in an animal which was breathing spontaneously and partly to the fact that they were not using single fibre preparations.

The physiology of stretch endings was investigated exhaustively by Adrian (8) and we have little to add to his findings. The fact that Adrian did not observe any increase in sensitivity of stretch endings with the only anaesthetic he used, namely chloroform, may be due to the transitory period of increased excitability produced by this drug. Matthews (10) observed a stimulating effect of chloroform and ether on stretch endings in mammalian striated muscle. Carlson (19) described a stimulating action of ether on the ganglion cells of *Limulus*, and Heinbecker and Bartley (12) showed that these cells are first stimulated and then paralysed by ether, but are paralysed by nembutal with little or no initial stimulation.

From our own results there can be little doubt that the volatile anaesthetics increase the excitability of the pulmonary stretch endings, and that this increased excitability is largely responsible for the reduction in the depth of respiration. The explanation of the changes in respiratory rate is a little more difficult. Trichlorethylene and cyclopropane have opposite effects on the respiratory rate at times when they both sensitise the endings to stretch. It follows that they must exert another action on a second set of pulmonary endings, or on the respiratory centre, or on extra-pulmonary endings. This last possibility may have to be reckoned with, since Hering (20) stated that 1%

chloroform stimulated carotid sinus mechanisms, whereas higher concentrations paralyse them (21). Paralysis of chemoreceptor reflexes by ether and cyclopropane, and stimulation by non-volatile anaesthetics has also been noted by Dripps and Dumke (22). A preliminary experiment on the administration of chloroform to the perfused sinus has confirmed these results, and has shown that here too the drugs act on the afferent nerve ending. This point needs further investigation. In all our decerebrate cats, however, both carotid arteries were tied, and effects on the carotid sinus were thus ruled out. A direct action of the anaesthetics on the respiratory centre was suggested by Heinbecker and Bartley (12), and this probably occurs with some anaesthetics, notably ether, but this effect is comparatively small.

It is possible to isolate one of the intra-pulmonary factors, other than stretch receptors, which affect rate and functional residual air during anaesthesia. The use of cold blocks to separate groups of fibres of different function in the vagus has been justified by the work of Hammouda and Wilson (14) and Partridge (16). These authors have established that the fibres which continue to conduct across a block at 5°C. are stimulated by deflation and cause inspiration. In the rabbit, we obtained evidence that the mechanisms responsible for the acceleration of the breathing during suction of air from the chest, are briefly stimulated by ether and then paralysed for the next 1-1½ hours, but are stimulated by trichlorethylene. Nevertheless, ether does not paralyse all the mechanisms responding to suction of air from the chest, as an increase in diaphragm tone persists even in deep ether anaesthesia. The increase in respiratory rate and in tone of the diaphragm is an immediate response to the suction, and is not due to remote effects on the blood pressure or the carotid body, since in the experiments of Head, Hammouda and Wilson and in our own, vagotomy or cooling to 1°C. abolishes it.

The consistent increase in functional residual air produced by trichlorethylene in decerebrate cats can be ascribed to a stimulation of deflation endings similar to that inferred in rabbits. The decrease in depth of respiration can be safely ascribed to the hyperexcitability of stretch endings, and the increase in rate of respiration is probably due to the cutting short of expiration as well as of inspiration.

With cyclopropane, the reduction in depth of respiration can again be attributed to moderate hyperexcitability of the stretch endings, but the factors responsible for the lengthening of the expiratory pause and the slowing of respiration have not yet been investigated.

All our experiments have been carried out on tracheotomised animals, and the results have therefore been simplified by the exclusion of the very important reflex effects initiated by irritation of the upper respiratory tract, which were studied by Magne, Mayer and Plantefol (23). Such effects of course play a dominant part in the earliest stages of induction. After these reflexes have disappeared, there is a very great similarity between the behaviour to anaesthetics of intact animals and the decerebrate tracheotomised animals. This makes it probable that the mechanisms which we have described account for the clinically familiar disturbances of respiration which are seen during anaesthesia, and in

particular for the rapid and shallow breathing which is so conspicuous with trichlorethylene.

SUMMARY

1 The effect of anaesthetics on the pulmonary afferent nerve endings has been investigated by recording action potentials in vagal single fibre preparations.

2. All volatile anaesthetics tested caused an increase in the sensitivity of stretch receptors. This occurred in spinal cats ventilated artificially with constant volumes of air, and also in decerebrate cats breathing spontaneously.

3. Cyclopropane and nitrous oxide caused hyperexcitability of stretch endings throughout exposure. Ethylchloride, chloroform, divinylether, ethyl ether and trichlorethylene caused stimulation followed by paralysis. Chloralose and nembutal caused no stimulation and but showed some depression in large doses.

4 The activity of deflation endings was studied in rabbits by selective blocking of the vagal fibres and recording from a diaphragm slip preparation.

5 Those deflation endings which produce acceleration of respiration in the rabbit, were first stimulated and then paralysed by ether, whereas trichlorethylene caused prolonged stimulation.

6 The interaction of these effects on stretch endings and on deflation endings, and the extent to which they determine the respiratory behaviour in the intact animal is discussed.

The authors wish to thank Mr W. T. S. Austin for technical assistance, and the Trustees of the Christopher Welch Fund for defraying the cost of the photographic materials used.

REFERENCES

- (1) DUNN, J S, *Quart J Med*, **13**, 129 (1919)
- (2) BINGER, C A L, BROWN, G R, AND BRANCH, A, *J Clin Invest*, **1**, 127 (1924)
- (3) CHRISTIE, R V, *Quart J Med*, **31**, 421 (1938)
- (4) KROHN, P L, WHITTERIDGE, D, AND ZUCKERMAN, S, *Lancet*, **1**, 252 (1942)
- (5) WHITTERIDGE, D, *J Sci Instrum* (in the press)
- (6) HAMILTON, W F, BREWER, G, AND BROTHMAN, I, *Amer J Physiol*, **109**, 427 (1931)
- (7) HEAD, H, *J Physiol*, **10**, 1 (1889)
- (8) ADRIAN, E D, *J Physiol*, **79**, 332 (1933)
- (9) ELFTUANN, A G, *Amer J Anat*, **72**, 1 (1943)
- (10) MATTHEWS, B H C, *J Physiol*, **78**, 1 (1933)
- (11) ADRIAN, E D, Unpublished observations (1944)
- (12) HILINBECKER, P, AND BARTLEY, S H, *J Neurophysiol*, **3**, 219 (1940)
- (13) McDOWALL, R J S, *Quart J Exp Physiol*, **16**, 291 (1927)
- (14) HAMMOUDA, M, AND WILSON, W H, *J Physiol*, **85**, 62 (1935)
- (15) CREED, R S, AND HERTZ, D H, *J Physiol*, **78**, 85 (1933)
- (16) PARTRIDGE, R C, *J Physiol*, **96**, 233 (1939)
- (17) KANDARACZI, M, *Pflüger's Arch*, **28**, 470 (1881).
- (18) KELLER, C H, AND LOFSER, A, *Z Biol*, **89**, 373 (1930)
- (19) CARLSON, A J, *Amer J Physiol*, **17**, 177 (1906)
- (20) HERRING, H E, *Die Karotissinusreflexe* Dresden u Leipzig (1927)
- (21) VERCAUTEREN, L, *Arch int Pharmacodyn Therap*, **42**, 339 (1932)
- (22) DRUMS, R D AND DUNN, P R, *This Journal*, **77**, 290 (1943)
- (23) MAGNE, H, MATYER, A, AND PLANTFOL, L, *Ann Physiol, Paris*, **1**, 509 (1925)

SOME COMPARATIVE PHARMACOLOGICAL ACTIONS OF BETA-HYDROXY AND METHOXY PHENYL-n-PROPYLAMINES

BOYD E. GRAHAM AND GEORGE F. CARTLAND

From the Research Laboratories, The Upjohn Company, Kalamazoo, Michigan

Received for publication May 12, 1944

Although the chemical, pharmacological and clinical literature on compounds chemically related to epinephrine, ephedrine and benzedrine is extensive, very little work has been reported on the β -phenyl-n-propylamines. The series here reported is, to our knowledge, the first complete β -hydroxy- and β -methoxy-phenyl-n-propylamine series with all the possible members having been synthesized and studied. Two members of this group have been previously prepared and only one of these tested pharmacologically, so far as we know. The nearest thing to a complete series of this type having been reported was the work of Hjort (1) on the N-methylated β -phenylethylamines.

β -phenyl-n-propylamine I was prepared by Hartung and Munch (2) and studied pharmacologically by Tainter (3), Schulte et al. (4), Warren et al. (5) and Beyer (6). It was also prepared by Hauschild (7) and preliminary toxicity tests were reported. The β -2,5-dimethoxyphenyl-n-propylamine (XII) was prepared by Baltzy and Buck (8) but no pharmacological studies appear in the literature.

This entire normal propylamine series was synthesized at these laboratories and the methods of preparation have been previously reported by Woodruff (9) and Woodruff and Pierson (10). This group of nineteen compounds includes the parent β -phenyl-n-propylamine and all the possible mono and dihydroxy, and mono and dimethoxy substitutions on the phenyl ring positions.

METHODS. The pharmacological studies made on this series of synthetic amines include acute toxicities, blood pressure determinations, and bronchiolar actions.

Acute Toxicity. White male rats with an average weight of 250 grams were used throughout this study. All toxicity figures represent the L.D. 50 in mgm. per kgm. of body weight. Solutions of the hydrochlorides were administered intravenously. They were made up in such concentrations that regardless of the size of the fatal dose, the injected volume per rat was always 1½ cc. or less. The possibility of manipulation error was minimized by maintaining this volume and holding the injection rate to not more than 0.25 cc. per minute. This appeared to be especially important with some of the more toxic members of the series.

The toxicity data summarized in Table I are based on a total of 438 rats. Each rat was used only once. After preliminary orientation tests not less than three groups of five rats each were used in the range of LD-50 \pm 10 mgm. per kilo; the exceptions being compounds IX and XIX. For compound IX, insufficient material was available to fix the upper limit. Lack of material prevented toxicity studies on compound XIX.

In general, the animals that died at or near the L.D. 50 did so immediately or within a few hours following administration, while those that survived one day post-injection invariably survived the fourteen day period of observation. In general, death from toxic doses was preceded by increased nervous excitability, increased respirations and heart rate, ruffled hair, profuse salivation in some cases, and finally, violent convulsions resulting in respira-

tory and cardiac failure. Animals surviving near toxic doses displayed most of these toxic manifestations but they were not nearly so pronounced.

Bronchial Action The bronchial action of these amines was measured on the lungs of freshly killed rabbits employing the method of Sollmann & von Oettinger (11). Fundamentally, this is a method of perfusing Locke's solution through the lung at a measurable rate. The rate of flow of fluid through the lung is indicated by the entrance of bubbles in the storage bottle of the closed system, as the fluid is displaced. In this work the normal or starting rate of flow was represented by 25 to 30 bubbles per minute. After preliminary studies with barium chloride, histamine, physostigmine and pilocarpine, the latter was chosen as the most satisfactory bronchoconstrictor for routine use in evaluating the members of this series. The sensitivity of the lung preparation was checked by the injection of epinephrine at the beginning and end of each experiment.

Not less than two lung preparations were used for each compound and in each case, intratracheal injections of the amine being tested were made in doses of 0.5, 1 and 2 cc. of 1 per cent aqueous solutions (5, 10 and 20 mgm.). Since the bronchial response was not always proportional to dosage, the effects produced by all three doses were averaged to obtain the bronchial ratings given in Table I which are recorded as average increases or decreases in the number of bubbles per minute.

Pressor Activity The blood pressure action of these compounds was studied in atropinized male dogs using morphine chloroform anesthesia. The direct method of recording arterial pressure as outlined in the U.S.P. was employed. All injections were made into the caudal right femoral vein and each was washed into the circulation with 2 cc. of physiological sodium chloride solution. Thirty-three dogs were used to evaluate the nineteen compounds. After a series of three to five responses to epinephrine, the amine being investigated was injected and only the first injection was considered in computing the ratio to epinephrine. It is believed that the pressor ratios estimated in this way are sufficiently accurate to characterize the amines.

RESULTS *β -phenyl n-propylamine I* was found to be about 1/500 as pressor as epinephrine. A 20 mgm. dose in a 14.8 kgm. dog produced a rise in arterial pressure of 50 mm. Hg. The pressure then gradually receded to a new normal level, 9 mm. above the former in fifteen minutes. This duration of pressor action closely agrees with the work of Fainter (3) in cats. The LD 50 was found to be 50 mgm. per kgm. On the isolated lung, this substance acted predominantly as a slight bronchoconstrictor hence has a bronchial rating of -2.

β -o-methoxyphenyl n-propylamine II is a depressor substance. A 10 mgm. dose produced a transient fall in arterial pressure of 15 mm. Hg. This depressor action agrees with the findings of Mulinos (13) in cats. As would be expected from its lack of pressor activity, Mulinos found this amine to be inactive in humans as a naso-mucous membrane vasoconstrictor using the improved nasograph mirror method of Lieb & Mulinos (14) originally reported by Glatzel (15). The addition of the ortho methoxy group to the parent substance I has reduced both the pressor activity and the acute toxicity. The LD 50 was found to be 80 mgm. per kgm. compared to 50 for the parent I. The bronchial activity was changed only slightly resulting in a bronchial rating for this substance of 0 or inactive.

β -o-hydroxyphenyl n-propylamine III differs from I by the addition of an OH group in the ortho position. This substitution has increased the bronchial action and the present derivative was found to be a bronchodilator with a rating of 3. The toxicity was further lowered and was found to be 110 mgm. per kgm.

The pressor action was considerably decreased over the parent I and is less than 1/5000 as effective as epinephrine. For all practical purposes, this o-hydroxy derivative may be said to possess little pressor activity. This lack of pressor

TABLE I

COMPOUND NUMBER	FORMULA	ACUTE TOXICITY L.D. 50-10% IN RATS MG./KG.	PRESSOR RATIOS IN DOGS-EPIN-1	BRONCHIAL RATING	REMARKS
I		50	1/500	-2	PROLONGED PRESSOR ACTIVITY
II		80	DEPRESSOR	0	NASOGRAPH TEST (14) NEGATIVE
III		110	LESS THAN 1/5000	3	NASOGRAPH TEST (14) NEGATIVE
IV		40	1/4000	8	
V		90	1/300	-1	
VI		30	1/2000	3	NASOGRAPH TEST (14) NEGATIVE
VII		170	1/2000	-2	PROLONGED PRESSOR ACTIVITY
VIII		30	INACTIVE	0	
IX		120+	LESS THAN 1/500	0	PROLONGED PRESSOR ACTIVITY
X		50	1/3000	8	
XI		150	1/1100	0	PROLONGED PRESSOR ACTIVITY
XII		50	1/2400	5	
XIII		100	LESS THAN 1/4000	0	
XIV		15	DEPRESSOR	4	
XV		90	DEPRESSOR	4	
XVI		140	1/675	1	
XVII		40	1/40	-2	NASOGRAPH TEST (14) 3+ DURATION-3 HRS +
XVIII		70	DEPRESSOR	8	
XIX		—	GREATER THAN 1/160	-1	

* NASOGRAPH TEST - 3+ = MAXIMUM NASAL CONSTRICTION

action is evidenced by nasograph studies by Mulinos (13) in which he found it inactive as a nasal vasoconstrictor in humans, and by Vaughan (16) in allergy patients in which it produced no increase in blood pressure when administered by mouth or subcutaneous injection in 25 mgm. doses.

β -m methoxyphenyl-n-propylamine IV compared to the parent I is only slightly more toxic acutely. The L D 50 was found to be 40 mgm per kgm. The blood pressure action was decreased eight fold, the ratio to epinephrine being 1/4000. The bronchodilator activity was considerably increased by this methoxy substitution. With a bronchial rating of 8 this derivative is one of the three most active bronchodilators of this n propylamine series.

β -m-hydroxyphenyl-n propylamine V This hydroxyl substitution caused the toxicity to be considerably decreased. The L D 50 was found to be 90 mgm per kgm. This is 40 mgm less than that of the parent I, and 50 mgm less than that of the corresponding methoxy member IV. The pressor activity has been increased to 1/300 that of epinephrine. The hydroxyl group in the meta position apparently does not improve the bronchial action over the unsubstituted parent as this substance (V) is a slight bronchoconstrictor and is rated at -1.

β p-methoxyphenyl-n-propylamine VI is the third mono methoxy derivative of this series. Compared to I the addition of the OCH₃ group to the para position has increased the toxicity from 50 to 30 mgm per kgm. The pressor action has been reduced four times to 1/2000 that of epinephrine. A 20 mgm dose in a 10.4 kgm dog produced a transient rise in arterial pressure of only 15 mm Hg. For all practical purposes this member has little pressor activity and Mulinos (13) found this true when he rated it ineffective as a nasal vasoconstrictor. This methoxy substitution improved the bronchial activity over I resulting in a bronchiolar rating of 3.

β -p-hydroxyphenyl n-propylamine VII is the OH derivative corresponding to VI. This p-hydroxy member is the third mono hydroxy of the series and has an L D 50 of 170 mgm per kgm. It is the least toxic of any of the nineteen compounds here reported. It is almost six times less toxic than the corresponding methoxy derivative and more than three times less toxic than the parent I. It is interesting to note here that an OH substitution in any of the three possible positions on the phenyl ring results in the substance becoming less toxic than the parent I. It was found to have a pressor ratio of about 1/2000 that of epinephrine. A 20 mgm dose in a 10.4 kgm dog produced a 20 mm rise in blood pressure that leveled off 16 mm above the former normal level and remained there for quite some time. It may therefore be rated as a prolonged pressor substance. It appears to have pressor activity similar to the corresponding methoxy derivative but possesses a more prolonged action. On the isolated lung it acted predominantly as a slight bronchoconstrictor and is rated at -2.

β 2,3-dimethoxyphenyl n propylamine VIII This derivative is one of the most toxic compounds of the series. Its L D 50 is 30 mgm per kgm, 20 mgm more toxic than the unsubstituted parent I. A 20 mgm dose intravenously in a 11.7 kgm dog produced no effect on the arterial pressure. Likewise, on the isolated rabbits lung it had no effect and consequently has a bronchial rating of 0.

β 2,3-dihydroxyphenyl n propylamine IX is one of the least toxic of this series. The toxicity is less than 120 mgm per kgm, but inadequate crystalline material made it impossible to further carry out this study. In contrast to the corre-

sponding dimethoxy member VIII, this dihydroxy compound has a greatly increased blood pressure action. Its ratio to epinephrine is slightly less than 1/500. A 20 mgm. dose in a 17.7 kgm. dog produced a 34 mm. rise in arterial pressure that gradually returned to normal in a period of twenty-one minutes. Hence, this substance may be said to possess a prolonged pressor action, although it finally does return to the pre-injection level. On the isolated lung it acted like the corresponding methoxy member, and is therefore rated 0, inactive.

β -2,4-dimethoxyphenyl-*n*-propylamine X is one of the three most effective bronchodilators of this series and is rated at 8. It is equally toxic with the parent I having an L.D. 50 of 50 mgm. per kgm. It is about 1/3000 as effective a pressor substance as epinephrine and for all practical purposes may be said to possess little pressor action.

β -2,4-dihydroxyphenyl-*n*-propylamine XI is similar to compound IX with respect to toxicity and bronchial activity. It was found to be inactive on the isolated lung and is therefore rated at 0. The L.D. 50 is 150 mgm. per kgm., making it one of the least toxic of the series. Its pressor ratio is 1/1100 that of epinephrine. A 10 mgm. dose in a 12.5 kgm. dog produced a rise in blood pressure of 18 mm. Hg., dropped to a level 10 mm. above the former normal and remained for about ten minutes. Although not a highly pressor substance, it does appear to have a somewhat prolonged action.

β -2,5-dimethoxyphenyl-*n*-propylamine XII is very much like X, the 2,4-dimethoxy derivative, pharmacologically. It has the same L.D. 50 of 50 mgm. per kgm. Its pressor ratio is about 1/2400, whereas X was about 1/3000. On the isolated lung, however, this 2,5-dimethoxy member is less active and has a bronchiolar rating of 5 compared to 8 for X.

β -2,5-dihydroxyphenyl-*n*-propylamine XIII is another one of the least active of the series. It has an L.D. 50 of 100 mgm. per kgm. and is less than 1/4000 as pressor as epinephrine. A 20 mgm. dose in a 16.4 kgm. dog produced a rise of only 13 mm. Hg. in blood pressure that was of short duration. On the isolated lung it was inactive and is therefore rated at 0.

β -2,6-dimethoxyphenyl-*n*-propylamine XIV is the most toxic compound of the entire series. Its L.D. 50 is 15 mgm. per kgm. It produced a transient fall in blood pressure and is therefore listed as a depressor substance. A 20 mgm. dose in a 13.8 kgm. dog caused a fall of 16 mm. Hg. in the arterial pressure. The bronchodilator activity was similar to that of the 2,5-dimethoxy XII derivative and it was given a bronchial rating of 4.

β -2,6-dihydroxyphenyl-*n*-propylamine XV is also a depressor substance of about the same caliber as its corresponding methoxy member (XIV) at a 20 mgm. dose in a 16.5 kgm. dog. Five mgm. doses produced no measurable effect. The bronchial action was the same also, as that of XIV. The toxicity, however, was considerably decreased compared to XIV. The L.D. 50 for this 2,6-dihydroxy member is 90 mgm. per kgm. or six times less toxic than the corresponding methoxy derivative.

β -3,4-dimethoxyphenyl-*n*-propylamine XVI, unlike the 2,6-dimethoxy, is one of the least toxic of the group. The L.D. 50 for this substance is 140 mgm. per

kgm Its pressor ratio is 1/675 and it is only slightly active on the isolated lung with a bronchial rating of 1

β-3,4-dihydroxyphenyl-*n*-propylamine XVII is by far the most potent pressor substance in this series and one of the most toxic It has a pressor ratio of 1/40 that of epinephrine, being more pressor than either epbedrine or benzedrine It appears to be more pressor than phenylpropanolamine (Propadrin) although it lies in that same range Mulinos (13) found this derivative, with the catechol nucleus, to be quite effective in humans as a naso-mucous membrane vasoconstrictor, with a duration of action over three hours The LD 50 was found to be 40 mgm per kgm On the isolated lung it was a bronchoconstrictor and is therefore rated at -2

β-3,5-dimethoxyphenyl-*n*-propylamine XVIII, like the 2,4-dimethoxy member, is one of the three most effective bronchodilators of this group of amines, and is given a bronchial rating of 8 The LD 50 was found to be 70 mgm per kgm A 20 mgm dose, in a 11.2 kilo dog produced a transient fall in arterial pressure of 22 mm of Hg It is therefore rated as a depressor substance

β-3,5-dihydroxyphenyl-*n*-propylamine XIX has much less action on the isolated lung than the corresponding methoxy derivative In fact, it was predominantly a bronchoconstrictor and has a rating of -1 As a pressor substance this 3,5-dihydroxy is second in potency only to the 3,4-dihydroxy member XVII It was found to have a pressor ratio of slightly greater than 1/160 that of epinephrine Compared to its corresponding methoxy derivative, the pressor activity has been greatly increased, while the bronchodilator activity was just reversed

GENERAL DISCUSSION The results as tabulated in Table I, show that the methoxy members of this *n*-propylamine series are more toxic intravenously in the rat than the corresponding hydroxyl derivatives There is one exception, this being in the case of the 3,4-dimethoxy compound XVI This substance might be expected to be quite toxic, but it was found to be the second least toxic of the series, while its hydroxyl derivative XVII was one of the most toxic

It is of interest to note that in general the bronchodilator activity appears to be best with the methoxy derivatives, although this too does not hold true in every case In all but three instances, the methoxy members are more effective bronchodilators than their corresponding hydroxyl derivatives The one actual reversal of this is in the *o*-methoxy II which is inactive as compared to the hydroxy compound III with a bronchial rating of 3 The other two exceptions are the 2,3 and 2,6 dimethoxy and hydroxy derivatives in which the corresponding members are of equal potency

As compared to the corresponding hydroxy members of the series, the methoxy derivatives are usually less pressor and exert a greater inhibitory effect on the bronchial musculature Vaughan (17) has reported positive clinical results with methoxy derivatives of another series The three most active bronchodilator compounds are methoxy derivatives with low pressor activity This lack of parallelism offers further evidence that these amines cannot be adequately evaluated by pressor ratios alone as has many times been reported in the older

literature. The work here reported indicates that high pressor ratios do not parallel the inhibitory effects on smooth muscle.

With reference to the position of the hydroxy group, we find that the meta hydroxy compound V is more pressor than the para compound VII. Barger and Dale (18) working with another series reported no difference. In agreement with the work of Schaumann (19, 20), Ehrhart (21) and Kuchinsky (22) on another series, we find that the meta hydroxy compound V is more pressor than either the ortho III or para VII. It also follows that the most active pressor substance of this group is the catechol nucleus member XVII. The intensifying effect of this configuration appears to come from the meta phenolic hydroxyl. This point is again brought out in the pressor action of the 3,5-dihydroxy member XIX, with a pressor ratio of greater than 1/160, the second most potent pressor substance of the series. Further evidence that the meta hydroxyl group is important and that hydroxyl groups in the 3,4 position lead to high pressor activity, are the results obtained with 2,3-dihydroxy derivative IX. One would expect IX to be less active on the blood pressure than the 3,4 dihydroxy XVII, since the single substituted o-hydroxy III is much less pressor than either the meta V or para VII members. Consulting Table I, we find this to be true.

SUMMARY

1. A complete series of β -phenyl-n-propylamines were studied pharmacologically with respect to their actions on the dogs blood pressure, isolated rabbits lung and acute intravenous toxicity in rats.

2. In general, the methoxy derivatives of this series were better bronchodilators than their corresponding hydroxyl analogues. Also, as a rule they were more toxic intravenously in rats.

3. In general, the hydroxy derivatives were more potent pressor substances than their corresponding methoxy analogues.

4. There appears to be a definite relationship between pressor action and naso-mucous membrane vasoconstriction as measured by the nasograph mirror method of Lieb and Mulinos (14).

5. No relationships could be demonstrated between bronchiolar and pressor actions.

REFERENCES

1. HJORT, A. M., *THIS JOURNAL*, 52: 101, 1934.
2. HARTUNG, W. H., AND MUNCH, J. C., *J. Am. Chem. Soc.*, 53: 1875, 1931.
3. TAINTER, M. L., *Arch. internat. de pharmacodyn et de therap.*, 46: 192, 1933.
4. SCHULTE, J. W., et al., *THIS JOURNAL*, 71: 62, 1941.
5. WARREN, M. R., et al., *THIS JOURNAL*, 79: 187, 1943.
6. BEYER, K., *THIS JOURNAL*, 71: 151, 1941.
7. HAUSCHILD, F., *Klin. Wchnschr.*, 20: 363, 1941.
8. BALTZLY, R., AND BUCK, J., *J. Am. Chem. Soc.*, 62: 161, 1940.
9. WOODRUFF, E. H., *J. Am. Chem. Soc.*, 64: 2859, 1942.
10. WOODRUFF, E. H., AND PIERSON, E., *J. Am. Chem. Soc.*, 60: 1075, 1938.
11. SOLLMANN, T. AND VON OETTINGEN, W. F., *Proc. Soc. Exper. Biol. and Med.*, 25: 692, 1928.

- 12 HARTUNG, W H , Chem Rev , 9 389, 1931
- 13 MULINGS, M G , Columbia Univ (personal communication)
- 14 LIEB, C C , AND MULINGS, M G , Arch Otolaryng 30 334, 1939
- 15 GLATZEL, Monatschr f Ohrench , Berl , 38 8-17, 1904
- 16 VAUGHAN, W T , Richmond, Virginia (personal communication)
- 17 VAUGHAN, W T , PERKINS, R M , DERNES, V J , J Lab & Clin Med 28 255, 1942
- 18 BARGER, G , AND DALE, H H , J Physiol , 41 19, 1910
- 19 SCHAUMANN, O , Arch f exper Path u Pharmacol , 157 114, 1930
- 20 SCHAUMANN, O , Arch f exper Path u Pharmacol , 160 127, 1931
- 21 EHRLHART, G , Metallboise, 20 1800, 1930
- 22 KUCHINSKY, G , Arch f exper Path u Pharmacol , 156 306, 1930

GENERAL ANALGESIC EFFECTS OF PROCAINE

NOLTON BIGELOW, M.D. AND IRVING HARRISON, M.D.

From the Departments of Medicine (Neurology) and Pharmacology, Cornell University Medical College, New York, New York

Received for publication May 18, 1944

It has long been recognized that cocaine produces not only local anesthesia but also possesses general analgesic properties (1). During the course of an investigation on certain aspects of the local anesthesia induced by procaine, it was noted that this drug, like cocaine, may produce general analgesia, and this action is the subject of the present report.

PROCEDURE AND METHOD. Measurements of the cutaneous pain threshold were made under various conditions on 5 subjects according to the technic described by Hardy, Wolff, and Goodell (2), which uses radiant heat as the source of painful stimulation. The subjects were carefully instructed as to the end-point, namely the first appearance of a pricking pain. The exact procedure deserves special note in view of the wide variations in normal pain thresholds observed by Chapman and Jones (3), when the subjects were not so carefully instructed as to the precise nature of the end-point.

Pain threshold determinations were made on the forehead; the injection of the agent to be tested was made subcutaneously in the arm. Control threshold measurements were made just prior to each injection and at approximately 10 minute intervals thereafter until the threshold returned to normal. All the subjects were found to have values for the control pain threshold well within the variation of ± 15 per cent reported by Schumacher, et al., for this technic (4). As a matter of fact, we found that under the conditions of our experiments in a group of 60 patients the threshold on the normally innervated forehead varied by not more than ± 10 per cent of the average for the series.

A 2 per cent solution of procaine hydrochloride was employed in doses ranging from 5 to 40 cc. injected subcutaneously. To evaluate the possible role of psychic factors in some experiments 5 or 10 cc. of physiologic saline solution was similarly given as a control, without the subject knowing the nature of the material injected.

In 3 experiments a perineural block of one ulnar nerve was produced by the injection of the procaine solution just above the elbow, for the purpose of comparing pain thresholds in the area subserved by the blocked nerve with those in the area subserved by the untreated ulnar nerve and on the forehead.

Not more than one experiment was carried out on a subject on any given day.

EFFECT OF SALINE CONTROL. In all cases some elevation of the pain threshold occurred after injection of 5-10 cc. of physiologic saline (figure 1). The average rise in threshold was not, however, as great as the average rise observed after the smallest dose of procaine, nor did the average elevation of threshold after physiologic saline persist as long as after procaine.

These results indicate that the injection of a presumably non-analgesic agent may so alter a subject's preceptual discrimination that a measurable elevation of the pain threshold occurs. Wolff and Goodell (5) also noted a rise in the pain threshold when a placebo was given to a suggestible subject. Conversely, they found no threshold rise in a suggestible subject who was told that the analgesic was a placebo. These observations make it imperative that in any study of the

analgesic potency of drugs, the factor of suggestion be taken into account and that control studies be made by the use of placebos or blanks, under conditions such that neither the experimenter nor the subject knows that a placebo is being used

In two subjects we gave large doses of physiological saline as controls for the large doses of procaine solution. In these experiments owing to the large volume of fluid injected associated with an absence of symptomatic effects, the subjects recognized that a placebo had been given. As might be expected in these instances, there were no changes in the pain threshold. Hence these data were not included in table 1.

GENERAL ANALGESIC ACTION OF INOCAINE Table 1 summarizes the changes in the pain threshold on the forehead after the injection in the arm of physiologic

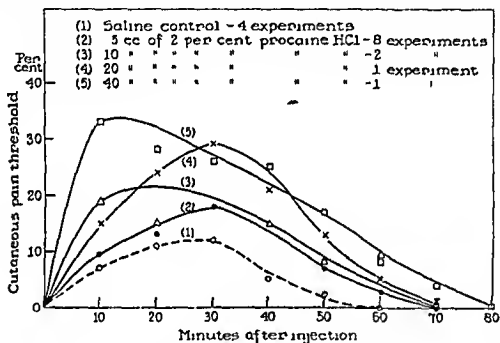


FIG. 1. GENERAL ANALGESIC EFFECT OF PROCAINE

saline and of procaine in various doses. Threshold measurements are tabulated to the nearest 10 minute interval after injection. In figure 1 the average rise in the pain threshold for each dose of procaine and for physiologic saline has been plotted. It may be seen that procaine raises the pain threshold on the forehead appreciably more than does the control injection of physiologic saline. The difference between the maximum rise produced by saline and by procaine was 5 per cent of the control threshold value for the 5 cc dose (100 mgm of procaine HCl), 8 per cent for the 10 cc dose (200 mgm of procaine HCl), 15 per cent for the 20 cc dose (400 mgm of procaine HCl), and 20 per cent for the 40 cc dose (800 mgm of procaine HCl). The average duration of the elevation in threshold after procaine was about 70 to 80 minutes, as compared with 50 to 60 minutes after saline. The maximum rise in the threshold after procaine was about 35 per cent above the patient's control threshold. This is approximately equivalent to the

maximum threshold-raising effect of acetylsalicylic acid: 0.3 gm. and 0.6 gm. doses of the latter both elevate the cutaneous pain threshold about 35 per cent, which thus represents the ceiling effect of this analgesic agent (6). In our series the number of experiments in the higher dosage range is insufficient to determine

TABLE 1

MATERIAL INJECTED	SUBJECT	CHANGE IN PAIN THRESHOLD AT VARIOUS INTERVALS AFTER INJECTION							
		10 min.	20 min.	30 min.	40 min.	50 min.	60 min.	70 min.	80 min.
		%	%	%	%	%	%	%	%
2% solution procaine HCl 5 cc.	N. H. B.	+2.6	+8.2	+15.6		+5.0	+0.7		
	N. H. B.	+34.2*	+32.4	+23.8	+12.3	+13.6	+3.4	-0.3	
	N. H. B.	+1.1	+5.2	+14.2	+7.1	+4.1			
	O. G. B.	-9.1	-18.6	+13.3	+6.1	+3.0			
	O. G. B.	+1.5	+7.0	+14.4*	+3.9	-0.6			
	I. B. H.	+12.7	+20.1	+25.2	+38.3*	+18.2	+11.2	+1.4	
	I. B. H.	+10.1	+5.6	+8.2	+5.0	+1.5	-0.4		
	I. B. H.	+5.2	+9.7	+29.0*	+16.6	+12.6			
	H. G.	+1.5	+13.7	+10.0	+8.9	+0.6	0.0		
	Averages†....	+8.6	+12.3	+17.6	+13.2	+6.9	+3.0	+0.6	
10 cc.	N. H. B.	+21.8*	+18.1		+29.1		+18.1	+0.4	
	N. H. B.	+21.4*	+12.4	+7.9	+2.3		-0.8		
Averages....		+21.6	+15.3		+15.2		+8.7	+0.4	
20 cc.	N. H. B.	+14.1	+23.1	+28.6*	+10.8	+12.1	+4.2		
40 cc.	N. H. B.	+32.8*	+27.7	+26.6	+25.4	+17.3	+7.7	+4.2	0.0
Physiologic saline solution	I. B. H.	+10.0	+7.1	+14.2	+4.1	+1.5	-0.4		
	I. B. H.	+2.6	+9.9	+12.2	+5.5	+0.7	-1.1		
5-10 cc.	N. H. B.	+12.1	+15.9		+5.3	+1.1			
	O. G. B.	+6.7	+12.6	+13.3	+5.6	+2.6	0.0		
Averages.....		+7.9	+11.4	+13.2	+5.1	+1.5	-0.8		

* Marked giddiness of subject.

† Excluding the results of the first experiment on O. G. B. from the average.

whether a threshold rise of 35 per cent represents the ceiling effect of procaine, or in fact, whether the general analgesic effect of this agent possesses such a ceiling. The duration of the procaine effect, about $1\frac{1}{4}$ hours, is much shorter than the duration of the effect of 0.3 gm. acetylsalicylic acid, namely $4\frac{1}{2}$ hours (6), although the total elevation of the pain threshold is about the same.

It is obvious that these changes in the pain threshold on the forehead can not be due to the local action of procaine injected in the arm, but are due rather to some systemic action of procaine after its absorption into the circulation. Gordon (7) has noted also a general analgesic action of procaine following its intravenous injection as demonstrated by the relief of pain in patients with burns.

CORRELATION OF ELEVATION OF PAIN THRESHOLD WITH OTHER GENERAL EFFECTS OF PROCAINE During the course of these experiments, the subjects sometimes reported sensations of light-headedness, giddiness or faintness, and once or twice, nausea. As table 1 indicates, the elevation in the pain threshold was usually greater when such effects were reported than when these were not noted. Moreover, the maximum rise in threshold usually occurred at the time when these sensations appeared. Thus, if the light headedness occurred within 10 minutes

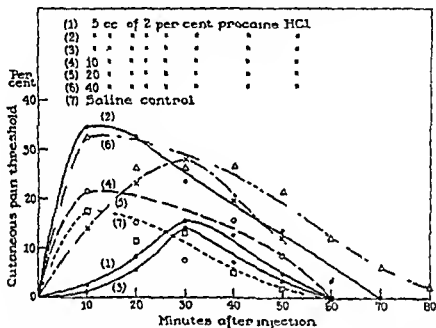


FIG. 2 VARIATIONS IN THE THRESHOLD RESPONSE TO PROCAINE IN ONE SUBJECT

after the injection, the maximum rise in the threshold also occurred at this time, whereas if the light headedness appeared after a longer interval, the maximum rise in threshold likewise occurred later. Thus it was noted that the maximum elevation of the pain threshold was apt to occur simultaneously with other central effects of procaine, and that the more pronounced were these other central effects, the greater was the rise in the pain threshold. This suggests that the general analgesic effect of procaine is produced by some central action.

VARIABILITY OF THRESHOLD RESPONSE. Figure 2 shows the effect on the pain threshold of various doses of procaine in the same subject and indicates that, at times, as great an effect may be obtained from 100 mgm. as from 800 mgm. of procaine hydrochloride. Thus, 5 cc. of the procaine solution raised the threshold to 34 per cent, whereas 10 cc. raised it to 32 per cent. In addition, repetition of the same dose (5 cc. of the 2 per cent procaine solution) in the same subject

maximum threshold-raising effect of acetylsalicylic acid: 0.3 gm. and 0.6 gm. doses of the latter both elevate the cutaneous pain threshold about 35 per cent, which thus represents the ceiling effect of this analgesic agent (6). In our series the number of experiments in the higher dosage range is insufficient to determine

TABLE 1

MATERIAL INJECTED	SUBJECT	CHANGE IN PAIN THRESHOLD AT VARIOUS INTERVALS AFTER INJECTION							
		10 min.	20 min.	30 min.	40 min.	50 min.	60 min.	70 min.	80 min.
		%	%	%	%	%	%	%	%
2% solution pro- caine HCl 5 cc.	N. H. B.	+2.6	+8.2	+15.6		+5.0	+0.7		
	N. H. B.	+34.2*	+32.4	+23.8	+12.3	+13.6	+3.4	-0.3	
	N. H. B.	+1.1	+5.2	+14.2	+7.1	+4.1			
	O. G. B.	-9.1	-18.6	+13.3	+6.1	+3.0			
	O. G. B.	+1.5	+7.0	+14.4*	+3.9	-0.6			
	I. B. H.	+12.7	+20.1	+25.2	+38.3*	+18.2	+11.2	+1.4	
	I. B. H.	+10.1	+5.6	+8.2	+5.0	+1.5	-0.4		
	I. B. H.	+5.2	+9.7	+29.0*	+16.6	+12.6			
	H. G.	+1.5	+13.7	+10.0	+8.9	+0.6	0.0		
	Averages†.	+8.6	+12.3	+17.6	+13.2	+6.9	+3.0	+0.6	
10 cc.	N. H. B.	+21.8*	+18.1		+29.1		+18.1	+0.4	
	N. H. B.	+21.4*	+12.1	+7.9	+2.3		-0.8		
Averages		+21.6	+15.3		+15.2		+8.7	+0.4	
20 cc.	N. H. B.	+14.1	+23.1	+28.6*	+19.8	+12.1	+4.2		
40 cc.	N. H. B.	+32.8*	+27.7	+26.6	+25.4	+17.3	+7.7	+4.2	0.0
Physiologic saline solution	I. B. H.	+10.0	+7.1	+14.2	+4.1	+1.5	-0.4		
	I. B. H.	+2.6	+9.9	+12.2	+5.5	+0.7	-1.1		
5-10 cc.	N. H. B.	+12.1	+15.9		+5.3	+1.1			
	O. G. B.	+6.7	+12.6	+13.3	+5.6	+2.6	0.0		
Averages		+7.9	+11.4	+13.2	+5.1	+1.5	-0.8		

* Marked giddiness of subject.

† Excluding the results of the first experiment on O. G. B. from the average.

whether a threshold rise of 35 per cent represents the ceiling effect of procaine, or in fact, whether the general analgesic effect of this agent possesses such a ceiling. The duration of the procaine effect, about $1\frac{1}{2}$ hours, is much shorter than the duration of the effect of 0.3 gm. acetylsalicylic acid, namely $4\frac{1}{2}$ hours (6), although the total elevation of the pain threshold is about the same.

It is obvious that these changes in the pain threshold on the forehead can not be due to the local action of procaine injected in the arm, but are due rather to some systemic action of procaine after its absorption into the circulation. Gordon (7) has noted also a general analgesic action of procaine following its intravenous injection as demonstrated by the relief of pain in patients with burns.

CORRELATION OF ELEVATION OF PAIN THRESHOLD WITH OTHER GENERAL EFFECTS OF PROCAINE During the course of these experiments, the subjects sometimes reported sensations of light-headedness, giddiness or faintness, and once or twice, nausea. As table 1 indicates, the elevation in the pain threshold was usually greater when such effects were reported than when these were not noted. Moreover, the maximum rise in threshold usually occurred at the time when these sensations appeared. Thus, if the light-headedness occurred within 10 minutes

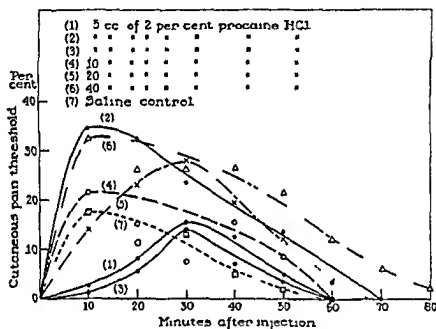


FIG. 2. VARIATIONS IN THE THRESHOLD RESPONSE TO PROCAINE IN ONE SUBJECT

after the injection, the maximum rise in the threshold also occurred at this time, whereas if the light-headedness appeared after a longer interval, the maximum rise in threshold likewise occurred later. Thus it was noted that the maximum elevation of the pain threshold was apt to occur simultaneously with other central effects of procaine, and that the more pronounced were these other central effects, the greater was the rise in the pain threshold. This suggests that the general analgesic effect of procaine is produced by some central action.

VARIABILITY OF THRESHOLD RESPONSE. Figure 2 shows the effect on the pain threshold of various doses of procaine in the same subject and indicates that, at times, as great an effect may be obtained from 100 mgm as from 800 mgm of procaine hydrochloride. Thus, 5 cc of the procaine solution raised the threshold to 34 per cent, whereas 40 cc raised it to 32 per cent. In addition, repetition of the same dose (5 cc of the 2 per cent procaine solution) in the same subject

on 3 different occasions produced a variable rise in the pain threshold, namely, from about 14 to 34 per cent. These differences may have been due to variations in the speed of absorption of the drug which is known to be an important factor in maintaining the systemic concentration of procaine because of the very rapid elimination of the drug (8, 9); or the differences may have been due to the psychic reaction of the subject at the time of the experiment.

In one subject the first administration of procaine was associated with an apparent lowering of the pain threshold. On a subsequent trial an elevation of the pain threshold comparable to the average rise was obtained. The reasons for the unusual response in this one instance are not entirely clear and are under investigation.

COMPARISON OF LOCAL AND GENERAL EFFECTS. Some observations were made as to the relative duration of the local and general analgesic actions of

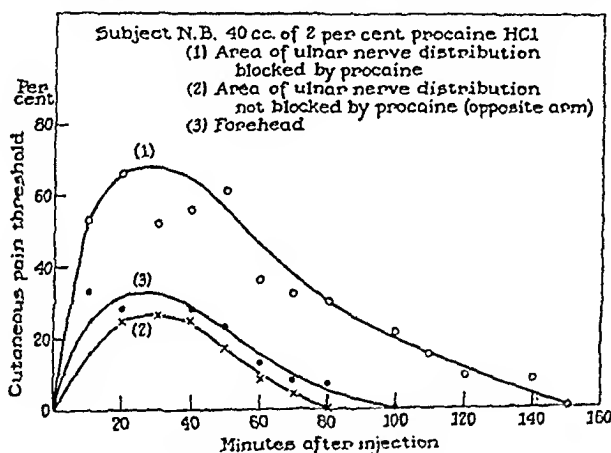


FIG. 3. COMPARISON OF THE DURATION OF THE GENERAL AND LOCAL ANALGESIC EFFECTS OF PROCAINE

procaine. In two experiments a perineural block of the ulnar nerve were so nearly complete that measurements of the pain threshold in the area of local anesthesia during the first hour after injection could not be made without damage to the tissues. However, in each case it was found that the pain threshold in the area of local anesthesia returned to normal about one hour after the general analgesic action had worn off.

In a third experiment a partial perineural block of the ulnar nerve was produced resulting in an elevation of the pain threshold in the hypesthetic area of only 68 per cent. Figure 3 contrasts the threshold measurements made on the forehead and on the ulnar aspects of the left and right hands subserved by the partially blocked and control ulnar nerves, respectively. It may be seen that the duration of the local action in this experiment also outlasts the general analgesic action of procaine by about one hour.

SUMMARY AND CONCLUSIONS

1. Procaine has a general analgesic action in addition to its well-known local anesthetic properties. The maximum rise in the cutaneous pain threshold attributable to the general action of this drug after 100 to 800 mgm. injected subcutaneously is approximately equivalent to the ceiling rise observed after acetylsalicylic acid, namely, about 35 per cent of the normal threshold value. The duration of the procaine effect is, however, much shorter than the acetylsalicylic acid effect.

2. The general analgesic action of procaine is usually more pronounced when other central effects of the drug are also evident.

3. The control injection of physiologic saline solution is also associated with a rise in the pain threshold, which, however, on the average is not as great as or prolonged as that observed after the smallest dose of procaine employed.

4. The local anesthetic action of procaine after a perineural block outlasts the general analgesic action of this drug by about one hour.

5. Variations are observed in the general analgesic effect of procaine on different occasions in the same subject independent of the dose.

BIBLIOGRAPHY

- (1) KAST AND MELTZER, *Medical Record*, 70: 1017, 1906.
- (2) HARDY, WOLFF AND GOODELL, *Jour. Clin. Invest.*, 19: 649, 1940.
- (3) CHAPMAN AND JONES, *Jour. Clin. Invest.*, 23: 81, 1944.
- (4) SCHUMACHER, GOODELL, HARDY, AND WOLFF, *Science*, 92: 110, 1940.
- (5) WOLFF AND GOODELL, *Proc. Assn. Res. In Nerv. and Ment. Dis.*, Williams & Wilkins, Baltimore, 1943, p. 434.
- (6) WOLFF, HARDY AND GOODELL, *Jour. Clin. Invest.*, 20: 63, 1941.
- (7) GORDON, *Canadian Med. Assn. J.*, 49: 478, 1943.
- (8) HATCHER AND FOOLESTON, *THIS JOURNAL*, 8: 335, 1916.
- (9) FOOLESTON AND HATCHER, *THIS JOURNAL*, 13: 433, 1919.

THE OXIDATION IN VITRO OF MORPHINE BY RAT LIVER SLICES

FREDERICK BERNHEIM AND MARY L. C. BERNHEIM

*From the Departments of Physiology and Pharmacology and of Biochemistry, Duke University
Medical School, Durham, N. C.*

Received for publication May 18, 1944

When morphine is administered to animals only a part of it is eliminated as such in the urine (1). In man (2) and dogs (3) some of the excreted morphine is in a conjugated form. In dogs the conjugation probably takes place in the liver for poisoning the animal with carbon tetrachloride reduces the amount of conjugated morphine excreted in the urine (3). The destruction of morphine also probably takes place in the liver for Inoue (4) recovered only 50% after incubating morphine with liver slices for 30 minutes. Simonnet (5) also claims that morphine disappears when incubated with liver and brain suspensions. It was therefore of interest to investigate this reaction more quantitatively in order to determine how the destruction of morphine occurs *in vitro*.

EXPERIMENTAL. Rat tissues were used for most of the experiments. They were sliced in the usual way and suspended in Krebs-Hensleleit solution (6) containing sodium bicarbonate but no glucose. Usually about 300 mgm. wet weight of tissue was shaken in 50 cc. Erlenmeyer flasks containing 4.0 cc. of the solution in equilibrium with 95% oxygen and 5% carbon dioxide. At the end of the experiment 1.0 cc. of 20% trichloroacetic acid was added and after the removal of the slices the precipitated proteins were centrifuged down. The morphine was estimated by adding 2.0 cc. of the silicomolybdic acid reagent, prepared according to the method described by Snell and Snell (7), followed by 10.0 cc. of 5% ammonium hydroxide. This reagent has the advantage of not being reduced by any of the constituents present in the trichloroacetic acid extract of slices when incubated aerobically. After anaerobic incubation a substance is present which causes a slight reduction of the reagent but this is not great enough to interfere with the estimation. Something is present in the extracts of liver suspensions which inhibits the reduction to some extent. The disadvantage of the reagent is that the color produced deviates from strict proportionality when more than 0.4 mgm. morphine is present. To overcome this difficulty a large number of blank vessels were run to which no morphine was added until just before the trichloroacetic acid. At this time graded amounts were added to different vessels which were then used as standards and the amounts remaining in the experimental vessels compared with them. The standards were chosen so that the colors developed in the experimental vessels gave values to within 10% of the standards. This procedure was also used for the anaerobic experiments and the experiments with tissue suspensions. The recoveries of added morphine obtained by this means averaged between 90-96%. Codeine and diionine although they precipitate with the reagent in acid solution give clear solutions with no color after the addition of the ammonium hydroxide. Dilaudid reduces the reagent and gives a blue color similar to that of morphine but weight

for weight the color is less than one half as intense. It was thus possible to follow the disappearance of dilaudid but the estimations were correspondingly less accurate. From these facts it appears that the silicomolybdic acid reagents is reduced by the two free hydroxy groups of morphine. If one of these groups is converted to a ketone as in dilaudid the reduction is decreased and if one of these groups is substituted by a methyl or ethyl group as in codeine and diionine no reduction occurs. Thus the disappearance of morphine as measured by the reduction of this reagent, when the drug is incubated with tissues, may be due either to the oxidation or conjugation of one or both of these hydroxy groups. Since these are important for the pharmacological action of morphine, a change in them would profoundly affect its action.

Table 1 shows that the liver slice under aerobic conditions is the only preparation that will effectively cause the disappearance of morphine *in vitro* under the experimental conditions. The reaction does not proceed anaerobically. This fact indicates that morphine does not disappear because of adsorption onto the slices or the protein precipitated by the trichloroacetic acid, because adsorption should occur anaerobically as well as aerobically. Also, the cell suspension

TABLE 1

The disappearance of 10 mgm morphine HCl when incubated 3-5 hours with tissues under various conditions

TISSUE	MORPHINE RECOVERED
	mgm
300 mgm liver slices (aerobic)	0.19
300 mgm liver slices (anaerobic)	0.82
300 mgm liver slices (aerobic, boiled)	0.96
500 mgm liver suspension (aerobic)	0.77
200 mgm kidney slices (aerobic)	0.85
500 mgm. brain suspension (aerobic)	0.94

offers a larger surface for adsorption and this preparation is much less effective in causing the disappearance of morphine. The cell suspensions of liver and brain were made with the least possible trauma and contained many apparently intact cells. The negative result with brain can therefore be considered significant for if this tissue could inactivate morphine it should have done so at least to the extent of the liver suspension. In our hands brain slices do not give reliable or reproducible results. The fact that slices boiled for 2 minutes in isotonic saline are inactive proves that the disappearance of morphine is caused by a thermolabile catalyst.

In order to determine whether the disappearance of morphine is due to the oxidation or conjugation of the hydroxy groups an aliquot was taken after removal of the proteins by trichloroacetic acid and placed in a boiling water bath for 30 minutes with N hydrochloric acid. The standards were subjected to the same treatment. This procedure hydrolyzes sulfates and glucuronates of phenolic compounds. The results were as follows. 20 mgm. of morphine HCl were added to 350 mgm of liver slices and after 4 hours incubation 0.46 mgm. were recovered. After boiling 0.43 mgm were recovered. In order to prove that

conjugation had not taken place earlier, to be followed by hydrolysis as the concentration of free morphine decreased, the experiment was repeated after 30, 60, and 90 minutes of incubation. In no case did boiling increase the amount of estimatable morphine. It therefore can be assumed that no conjugation occurs and that the hydroxy groups are oxidized. The time curves for the oxidation of 1.0 and 2.0 mgm. of morphine HCl are given in Fig. 1. These experiments were repeated with dilaudid with the same results. Dilaudid is oxidized at approximately the same rate as morphine.

Since codeine and dionine give no color with the reagent and since their presence in at least ten times the concentration does not interfere with the morphine estimation, it was possible to study the effect of these compounds on the oxidation of morphine by liver slices. Control experiments showed, how-

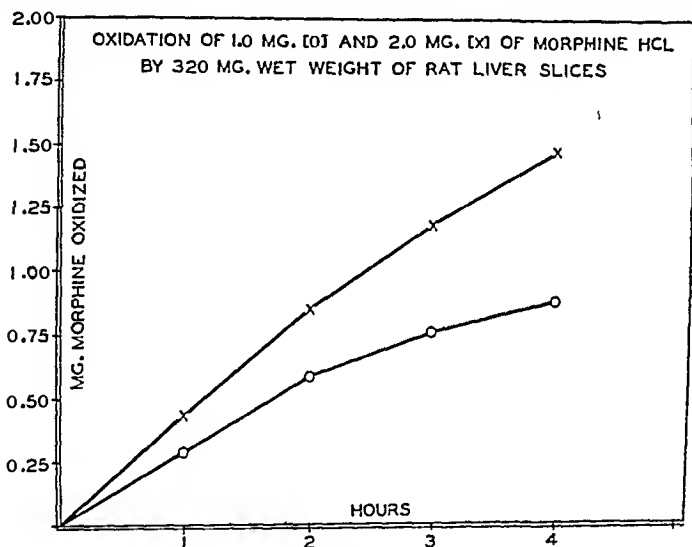


Fig. 1

ever, that both codeine and dionine after incubation with liver slices aerobically will reduce the silicomolybdic acid reagent to give the same color as morphine. Thus after 3.5 hours incubation of 1.0 mgm. codeine with 300 mgm. liver slices a color is produced that is equivalent to 0.13 mgm. morphine. From 2.0 mgm., 0.20 mgm., and from 3.0 mgm., 0.22 mgm. is produced. The corresponding values for 1.0 mgm., 2.0 mgm., and 3.0 mgm. dionine are; less than 0.08 mgm., 0.15 mgm., and 0.16 mgm. The production of this color can be due to the demethylation and de-ethylation of codeine and dionine respectively, or the presence of these compounds causes the liver to form a substance that will reduce the reagent. To distinguish between these two possibilities is difficult. The breaking of ether linkages such as those in codeine and dionine has not been reported to occur in the animal body. It would therefore be premature to attribute this color production to the conversion of codeine and dionine to mor-

phine On the other hand, one would expect a) that if eodine and dionine produced some reducing substance in liver morphine would also have some effect, and b) if such a substance were produced, tripling the codeine concentration would increase the amount formed more than 0.08 mgm. In no case was the color produced equivalent to more than 15% of the eodine or dionine added. Anaerobically no color is produced from these two compounds. Aerobically the brain produces none and the kidney only a trace.

Despite this complication, it was possible to show that the presence of codeine or dionine apparently inhibited the oxidation of morphine by liver slices. Thus after 3.5 hours incubation 0.21 mgm. morphine was recovered out of 1.0 mgm. morphine HCl added. 1.0 mgm. codeine phosphate produced the equivalent of 0.13 mgm. morphine. When 1.0 mgm. codeine and 1.0 mgm. morphine were incubated together the equivalent of 0.53 mgm. morphine was recovered. If the codeine had merely added its effect then an equivalent of 0.34 mgm. morphine would have been obtained. Consequently it is possible to assume that morphine is oxidized by a specific enzyme for which the codeine is competing rather than by, for instance, hydrogen peroxide formed as a by-product of the oxidation of some substrate in the liver.

Guinea pig liver will also oxidize morphine but more slowly than rat liver. After 3.5 hours incubation only 0.4 mgm. was oxidized out of 1.0 mgm. morphine HCl added to 300 mgm. liver slices, whereas under the same conditions rat liver will oxidize 0.8-0.9 mgm. Since the M.L.D. of morphine for the guinea pig is almost twice that for the white rat the rate of oxidation apparently plays no direct part in determining the dose tolerated. This must depend on the distribution of the drug in the body and the amount stored in tissues such as muscle.

The above experiments indicate one of the means by which morphine can be altered in the body but do not elucidate the mechanism of its conjugation.

SUMMARY

- 1 Morphine added to rat liver slices is oxidized apparently at the hydroxy groups. Kidney and brain are inactive.
- 2 The reaction does not occur under anaerobic conditions or after boiling the slices. Liver cell suspensions oxidize morphine much more slowly than slices.
- 3 No evidence of conjugation was found.
- 4 Dilaudid is also oxidized under the same conditions.
- 5 Codeine and dionine apparently inhibit the oxidation of morphine.

REFERENCES

- (1) GOODMAN AND GILMAN, *The Pharmacological Basis of Therapeutics*, p. 202, 1941. The MacMillan Co.
- (2) ONFRST, *This Journal*, 73, 401, 1911.
- (3) GROSS, *Proc. Soc. Exptl. Biol. and Med.*, 51, 61, 1912.
- (4) INOUE, *Jap. J. Med. Sci. & Pharmacol.*, 12, 1910.
- (5) SIMONET, *Compt. Rendus*, 204, 1371, 1937.
- (6) KRERS AND HENSELEIT, *Z. Physiol. Chem.*, 210, 33, 1932.
- (7) SNELL AND SNELL, *Colorimetric Methods of Analysis II*, p. 510, 1936. Van Nostrand.

THE DIGITALIS CAT ASSAY IN RELATION TO RATE OF INJECTION

C. I. BLISS

Yale University and Connecticut Agricultural Experimental Station

AND

M. G. ALLMARK

Laboratory of Hygiene, Department of Pensions and National Health, Canada

The assay for digitalis adopted in the U.S.P. XII calls for the injection of a diluted tincture into the femoral vein of the cat until the heart stops beating. The injection fluid is to contain the estimated fatal dose per kg. in each 15 cc., and it is injected intermittently at the rate of one cc. per kg. every 5 minutes until the end point is reached. Ideally the number of injections in a group of cats should average 15 but the Pharmacopoeia permits assays with an average of 13 to 19 injections per group. How great an error may be introduced in the estimate of potency by a range of this magnitude?

Vos and Dawson (4) have reported that in the range of their tests the fatal dose decreased as the rate of injection was lowered. Moreover, with some cardiac glycosides they obtained more precise measurements by adjusting differences in body weight by covariance rather than by the commonly assumed relation of mg./kg. In unpublished experiments with guinea pigs at Ottawa, Chapman observed a similar change in the lethal dose of tincture as the concentration of the injection fluid was increased. No experiments have been reported as yet, however, with the periodic injection technique of the U.S.P. XII assay. The present study was undertaken to fill this gap by determining whether the concentration of tincture affects the average lethal dose in the U.S.P. XII procedure and if a range of concentrations other than 13 to 19 should be used.

EXPERIMENTAL METHODS AND RESULTS. Three experiments have been run, each with a different commercial sample of digitalis powder which satisfied the requirements of U.S.P. XI as standardized on frogs. Tinctures were prepared from the powders by the method developed in the collaborative digitalis assays (1) and later adopted by the Pharmacopoeia. Prior to each experiment, pilot tests determined the concentration of injection fluid which would kill in 14 to 18 injections at five-minute intervals. This concentration was designated as 100 per cent and three additional solutions were made representing respectively 125, 80, and 64 per cent of this quantity. In every case the solutions for injection were prepared from the same undiluted tincture on the day of the test. Each was injected at the rate of one cc. per kg. every 5 minutes until the heart stopped.

The experiments were made upon groups of four cats, each cat in a group receiving a different one of the four dilutions. With one exception, all cats in a group were injected on the same day and in most cases it was possible to run two groups a day. The cats forming a group were selected for similarity in weight, length of time held in the laboratory and the source from which they were ob-

tained. The cats varied in weight from 1.7 to 4.2 kg but only 15 of the 144 cats exceeded the Pharmacopoeial limits of 2.0 to 4.0 kg. Since the lethal doses for the outsize individuals were consistent with those for the other cats, they have been retained in the analysis.

Each experiment comprised 12 groups of four animals. The first experiment was completed during the period from February 10 to 17 inclusive, the second from February 18 to 25 and the third from February 25 to March 4, all in 1942, at the Laboratory of Hygiene in Ottawa, Canada. Ether served as the anaesthetic and unless otherwise specified, the conduct of the assays followed the procedure described in U S P XII.

The experimental results have been summarized in table 1, where each row lists the data for 12 cats. The mean number of doses (at five minute intervals)

TABLE 1

Summary of the original data of three experiments on the biological assay of digitalis in cats, 12 cats in each row

EXPERIMENT AND DATE 1942	TINCTURE IN 100 CC. OF INJECTION FLUID	GEOMETRIC MEAN WT. OF CATS	AVERAGE NO. OF DOSES	GEOMETRIC MEAN SURVIVAL TIME	LD50 OF TINCTURE	STANDARD DEVIATION
	cc	kg		min	cc/kg	log-dose
I 2/10-2/17	2.94	2.73	26.2	128.3	7646	0621
	3.63	2.83	21.4	104.1	7842	0436
	4.60	2.81	17.2	83.1	7830	0607
	5.75	2.78	15.3	73.9	8738	0620
II 2/18-2/25	2.56	2.85	21.2	104.0	5421	0414
	3.20	2.89	19.3	92.4	6079	0340
	4.00	2.83	14.5	68.6	5704	0329
	5.00	2.86	11.6	55.5	5763	0505
III 2/25-3/4	2.56	2.66	21.2	102.7	5373	0621
	3.20	2.65	17.3	84.3	5520	0454
	4.00	2.71	16.5	79.0	6491	0328
	5.00	2.68	13.6	65.5	6705	0747

is an arithmetic average, the other three averages are geometric means or the antilogarithms of the mean of the log weight, of the log minutes survival, and of the log dose, respectively. The antilogarithm of the mean log dose is presumably the best estimate of the LD50 and is so named in the table. The significance and interrelations of these values will be developed in the rest of the paper.

THE CORRECTION FOR SIZE. In the cat assay for digitalis differences in size are adjusted (a) by injecting digitalis at one cc. of dilute solution per kg. of body weight and (b) by expressing the fatal dose in cc. per kg. With some cardiac glycosides, this has tended to overcorrect differences in weight (4), although with digitalis it has proved as satisfactory as less arbitrary alternatives (2). In the present data the body weight differed significantly between groups. However these differences did not affect the lethal dose significantly when ex-

THE DIGITALIS CAT ASSAY IN RELATION TO RATE OF INJECTION

C. I. BLISS

Yale University and Connecticut Agricultural Experimental Station

AND

M. G. ALLMARK

Laboratory of Hygiene, Department of Pensions and National Health, Canada

The assay for digitalis adopted in the U.S.P. XII calls for the injection of a diluted tincture into the femoral vein of the cat until the heart stops beating. The injection fluid is to contain the estimated fatal dose per kg. in each 15 cc., and it is injected intermittently at the rate of one cc. per kg. every 5 minutes until the end point is reached. Ideally the number of injections in a group of cats should average 15 but the Pharmacopoeia permits assays with an average of 13 to 19 injections per group. How great an error may be introduced in the estimate of potency by a range of this magnitude?

Vos and Dawson (4) have reported that in the range of their tests the fatal dose decreased as the rate of injection was lowered. Moreover, with some cardiac glycosides they obtained more precise measurements by adjusting differences in body weight by covariance rather than by the commonly assumed relation of mg./kg. In unpublished experiments with guinea pigs at Ottawa, Chapman observed a similar change in the lethal dose of tincture as the concentration of the injection fluid was increased. No experiments have been reported as yet, however, with the periodic injection technique of the U.S.P. XII assay. The present study was undertaken to fill this gap by determining whether the concentration of tincture affects the average lethal dose in the U.S.P. XII procedure and if a range of concentrations other than 13 to 19 should be used.

EXPERIMENTAL METHODS AND RESULTS. Three experiments have been run, each with a different commercial sample of digitalis powder which satisfied the requirements of U.S.P. XI as standardized on frogs. Tinctures were prepared from the powders by the method developed in the collaborative digitalis assays (1) and later adopted by the Pharmacopoeia. Prior to each experiment, pilot tests determined the concentration of injection fluid which would kill in 14 to 18 injections at five-minute intervals. This concentration was designated as 100 per cent and three additional solutions were made representing respectively 125, 80, and 64 per cent of this quantity. In every case the solutions for injection were prepared from the same undiluted tincture on the day of the test. Each was injected at the rate of one cc. per kg. every 5 minutes until the heart stopped.

The experiments were made upon groups of four cats, each cat in a group receiving a different one of the four dilutions. With one exception, all cats in a group were injected on the same day and in most cases it was possible to run two groups a day. The cats forming a group were selected for similarity in weight, length of time held in the laboratory and the source from which they were ob-

tained. The cats varied in weight from 1.7 to 4.2 kg but only 15 of the 144 cats exceeded the Pharmacopoeial limits of 2.0 to 4.0 kg. Since the lethal doses for the outsize individuals were consistent with those for the other cats, they have been retained in the analysis.

Each experiment comprised 12 groups of four animals. The first experiment was completed during the period from February 10 to 17 inclusive, the second from February 18 to 25 and the third from February 25 to March 4, all in 1942, at the Laboratory of Hygiene in Ottawa, Canada. Ether served as the anesthetic and unless otherwise specified, the conduct of the assays followed the procedure described in U.S.P. XII.

The experimental results have been summarized in table 1, where each row lists the data for 12 cats. The mean number of doses (at five minute intervals)

TABLE 1

Summary of the original data of three experiments on the biological assay of digitalis in cats, 12 cats in each row

EXPERIMENT AND DATE 1942	TINCTURE IN 100 CC. OF IN- JECTION FLUID	GEOMETRIC MEAN WT. OF CATS	AVERAGE NO. OF DOSES	GEOMETRIC MEAN SUR- VIVAL TIME	LD50 OF TINCTURE	STANDARD DEVIATION
	cc	kg		min	cc/kg	log-dose
I 2/10-2/17	2.94	2.73	26.2	128.3	7646	0621
	3.68	2.83	21.4	104.1	7842	0436
	4.60	2.81	17.2	83.1	7830	0607
	5.75	2.78	15.3	73.9	8738	0620
II 2/18-2/25	2.66	2.85	21.2	104.0	5421	0414
	3.20	2.89	19.3	92.4	6079	0540
	4.00	2.83	14.6	68.6	5704	0829
	6.00	2.86	11.6	55.5	6763	0505
III 2/25-3/4	2.66	2.66	21.2	102.7	5373	0621
	3.20	2.65	17.3	84.3	5520	0454
	4.00	2.71	16.6	79.0	6491	0828
	5.00	2.68	13.6	65.5	6705	0747

is an arithmetic average. The other three averages are geometric means or the antilogarithms of the mean of the log weight, of the log minutes survival, and of the log dose, respectively. The antilogarithm of the mean log-dose is presumably the best estimate of the LD50 and is so named in the table. The significance and interrelations of these values will be developed in the rest of the paper.

THE CORRECTION FOR SIZE. In the cat assay for digitalis differences in size are adjusted (a) by injecting digitalis at one cc of dilute solution per kg of body weight and (b) by expressing the fatal dose in cc per kg. With some cardiac glycosides, this has tended to overcorrect differences in weight (4), although with digitalis it has proved as satisfactory as less arbitrary alternatives (2). In the present data the body weight differed significantly between groups. However, these differences did not affect the lethal dose significantly when ex-

pressed in cc. per kg. Examination by covariance revealed no better way of adjusting for body weight than to use the simple ratio of cc. per kilogram as the measure of the lethal dose.

ANALYSIS OF THE VARIANCE BETWEEN INDIVIDUAL LETHAL DOSES. Three experiments tested the dependence of the lethal dose upon the concentration of tincture in the injection fluid, which in turn, governed the rate of injection. This relation and the effect of other variables in the experiment were examined statistically by the analysis of variance. Since the logarithm of the lethal dose of digitalis in cats has been shown to follow the normal distribution (1), the individual doses were expressed in logarithms for calculation. The results from the three series are given in table 2.

The first two rows in Table 2 test whether the different samples of drug used in the three experiments were of equal potency. Since these samples had been standardized on frogs, this was a comparison in effect of their relative potency in two different species. Without adjustment for rate of injection, the preparation

TABLE 2

Combined analysis of variance for the three experiments, computed in terms of the individual log-dose of original tincture

TERM	DEGREES OF FREEDOM	MEAN SQUARE	VARIANCE RATIO F	
Experiment I vs. II and III.....	1	.583560		127.11
Experiment II vs. III.....	1	.008702		1.90
Between 4-cat groups.....	33	.004591	1.15	1
Average slope of log-dose on log-concentration...	1	.068289	17.06	
Differences in slope between experiments.....	2	.014330	3.58	
Curvature.....	6	.004583	1.15	
Error within groups.....	99	.004002	1	

in Experiment I was only 73 per cent as potent as the average of those used in Experiments II and III, which latter were of comparable potencies. These conclusions are supported by the highly significant variance ratio in the first row of table 2 ($F = 127.11$) and the small ratio in the second row. These samples were tested in succession rather than simultaneously, so that the variation between four-cat groups is here the appropriate error term. Because of the experimental design, the overall difference in potency between the three preparations did not bias the other comparisons in the table.

The next factor of interest is the stability of the variance, which would determine the most suitable term for the error in later comparisons. Although the cats in each group were selected for their similarity, in the 8 days covered by each series this procedure did not segregate any substantial differences in susceptibility to digitalis. The variance between groups exceeded that within groups by only 15 per cent ($F = 1.15$), much less than would be required for statistical significance. This agrees with the results of the U.S.P. collaborative cat assays (1). Since the mean square between 4-cat groups in table 2 agreed so nearly

with that for the error within groups, differences between days were disregarded in computing the standard deviation from the 12 log doses for each rate of injection in each series. These have been listed in the last column of table 1. The standard deviations showed no consistent trend related to rate of injection and did not differ more from one another than might be expected by chance ($\chi^2 = 14.56$, $n = 11$, $P = 0.20$). In other words, differences in the rate of injection did not affect the precision of the test. The combined value for all estimates of the standard deviation was $s = 0.06442$ with 132 degrees of freedom.

The remaining terms in table 2 concern the main objective of the study: was the fatal dose of digitalis related to rate of injection within the present range of concentrations? Taking the three experiments as a whole, the mean log dose required to stop the heart increased very significantly with the rate of administration as controlled by the concentration of the injection fluid. This was determined from the variation attributable to the average slope of the straight lines relating log dose to log concentration ($F = 17.06$, $P < 0.001$). The relation between these two variables could be fitted adequately by straight lines, since the mean square in Table 2 for curvature or non linearity was very close to that for error ($F = 1.15$). However, the straight lines for the three experiments diverged from one another more than would be expected by chance for parallel lines ($F = 3.58$, $P < 0.05$). Between Experiments I and III the difference in slope was not significant, but in Experiment II the trend of log dose upon log concentration was negligible and hence significantly less than the average for the other two. Thus the analysis in table 2 has shown that within the range of concentrations in the present experiments the mean lethal dose of digitalis may depend upon the rate of injection.

EFFECT OF CONCENTRATION UPON THE OFFICIAL ASSAY The analysis in table 2 has established the existence of a linear relation between log dose and the log concentration of tincture in the injection fluid. The fact that the three experiments differed in slope would lead to caution in assessing the average magnitude of this effect. In order to see how the individual means are distributed about the average slope, the differences in the potency of the three samples have been adjusted to a common basis and plotted in figure 1. The basis for this adjustment will be discussed in a later section. Since the scatter of the plotted points about the computed line indicated a somewhat greater consistency than would be judged from the analysis of variance alone, the combined regression coefficient of $b_c = 0.201 \pm 0.053$ has been used as the best available estimate for computing the potential bias in a U.S.P. assay.

The Pharmacopoeia requires that the dilute solution must be of such a concentration that the number of five minute injections required to reach the end point shall average not less than 13 nor more than 19 in any acceptable group. Let us suppose that these extremes were to occur in a single assay, the reference standard averaging 13 injections and the unknown 19 injections. Assuming a direct equivalence between the two quantities of digitalis, the potency of the unknown would be $\frac{13}{19} = 0.6842$ that of the standard by U.S.P. definition. However, this has not been corrected for the relation between lethal dose and survival

time, and relatively less of the unknown has had to be injected to produce a kill in the larger number of injections.

The correction may be based upon a linear relation between the log-dose observed experimentally and the true log-concentration of injection fluid with a slope of $b = 0.20$ as in figure 1. Then if the concentration of the unknown were increased to the point where it would produce death in 13 injections, the required concentration in logarithms would be, not $\log \frac{1}{13}$ as expected if the concentration of the dilute solution had no effect, but $\log \frac{1}{13} + 0.20 \log \frac{1}{13}$ or $.1648 + .0330$. In original units the expected dose would be multiplied by the antilogarithm of .0330 or by 1.079. Hence the limits of 13 and 19 in the mean number of injections could lead to a bias of about 8 per cent in the estimated potency of the unknown. Since 8 per cent more of the weak, unknown tincture would be required

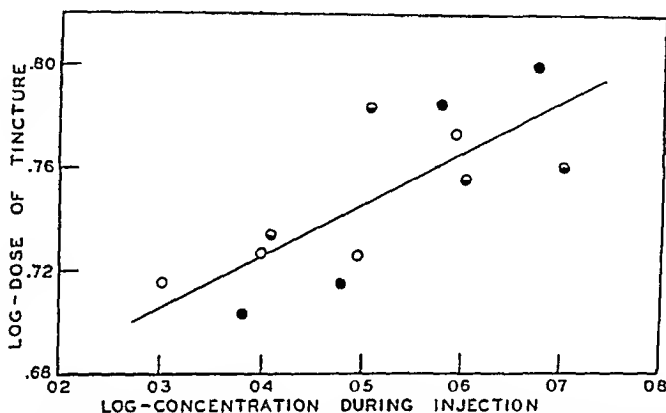


FIG. 1. RELATION BETWEEN FATAL DOSE OF TINCTURE OF DIGITALIS AND ITS CONCENTRATION IN THE INJECTION FLUID, BOTH IN LOGARITHMIC UNITS

The results for three tinctures, identified by different symbols, have been adjusted to the same potency, as described in the text

than expected from the ratio of $\frac{1}{13}$ to produce death in the same 13 doses as the standard, its true potency would be overestimated by the hypothetical assay just described. Conversely, the true potency of strong tinctures would be underestimated.

In practice, of course, such extreme differences would rarely occur. Adjustments of the concentration of the injection fluid based upon pilot tests usually would have reduced the difference by half or more, and eliminated the correlation between the true potency of the tincture and its relative dilution in the injection fluid. Hence it is likely that the bias would seldom exceed 4 per cent of the estimated potency.

AN ALTERNATIVE CRITERION FOR EVALUATING POTENCY. In view of the dependence of the lethal dose upon the rate of injection, a method of assay would be preferred that depended less upon one's success in predicting the potency of the

unknown. The three experiments in the present study, for example, were made with tinctures of different potency. In assaying their relative values, should one base his estimates only upon those groups of 12 where the average number of injections fell within the pharmacopoeial limits of 13 to 19? In such a case the relative potency of the powder in Experiment I would be determined from the results of two dilutions, that in Experiment II from one dilution and that in Experiment III from three dilutions (see table 1). An estimate of relative potency based upon all of the data would be more reliable statistically than one computed from only part of the evidence.

This requirement can be met by using the logarithm of survival time as the response. Survival time was recorded in minutes for each cat, independently of the number of injections. It has the advantage of being measured on a relatively continuous scale instead of one divided in intermittent parts. If the cat dies during an injection, the question does not arise as to whether the fatal dose should or should not include this last portion. Because survival time is so closely linked to the fatal dose, both involve the same pharmacological response and potency computed from one should have the same clinical value as that from the other. Since the logarithm of the dose has been shown to follow the normal distribution, the logarithm of survival time would be expected to do the same, so that minutes have been changed to logarithms for the present criterion of response.

The calculation of potency and its error in an assay based upon survival time closely parallels that involving a true graded response, so that methods suitable for the latter (3) have been applied here with little change. The symbol y has been defined as the response in log minutes and x as the log concentration of the injection fluid. The data for each experiment have been computed in two ways. (1) All observations were included in a single dosage response curve with four equally spaced log concentrations or doses. (2) The same data were computed as an assay on the assumption that the first and third concentrations represented the "standard" and the second and fourth concentrations an "unknown."

THE CONCENTRATION RESPONSE CURVE FOR DIGITALIS. The results of calculation as a dosage response curve are given for each experiment separately in Table 3. The relative importance of the different sources of variation may be judged from the mean squares in the analysis of variance of the first four rows. In the first experiment the variation between four cat groups was less than its error—the interaction of groups by doses—and in the second experiment the relation was reversed, with both apparently significant ($P < 0.05$). This reversal and the equivalence of the two terms in Experiment III suggested that over the three experiments the variances between and within groups might have differed only by the error of sampling. The six variances were then tested for homogeneity by χ^2 , which showed that the differences between them could be attributed to chance ($\chi^2 = 8.74, n = 5, P \approx 0.12$). Hence both the differences between four cat groups and the interaction of groups by doses have been combined in computing for each experiment the standard deviation in response

(s). The variance (B^2) attributable to the slope of a straight line relating log-time (y) and log-dose (x) was much the largest term in all experiments. The small variances for curvature indicate that the relation between x and y could be fitted adequately by straight lines.

The precision which may be expected in bioassays based on the logarithm of survival time is determined by the standard deviation of the log-dose as estimated from the dosage-response curve. This is equal to the ratio of the standard deviation (s) to the slope of the curve (b) or

$$\lambda = \frac{s}{|b|} \quad (1)$$

TABLE 3

Analysis by separate experiments of log-minutes survival time with a single dosage-response curve computed from all four dilutions or doses

	D. F.	RESULTS FROM EXPERIMENT NO.		
		I	II	III
Variance or mean square for				
Differences between 4-cat groups...	11	.00158	.00907	.00452
Slope of dosage-response curve (B^2)...	1	.39886	.53913	.22614
Curvature about straight line.....	2	.00326	.00649	.00365
Interaction of groups by doses.....	33	.00433	.00357	.00507
Statistics from dosage-response curve				
Standard deviation in response, s ...	44	.06037	.07031	.07025
Slope of curve, b		-.8413	-.9781	-.6335
Standard deviation in log-dose, λ0718 \pm .0103	.0719 \pm .0103	.1109 \pm .0202
Mean response, \bar{y}9785	.8909	.9129
Mean log-dose, \bar{x} (+2).....		.6143	.5536	.5536

where $|b|$ indicates that here the slope is taken as positive. By transformation of Equation (11) in reference (3), the standard error of λ may be computed as

$$s_{\lambda} = \sqrt{\frac{1}{2n} + \frac{s^2}{B^2}} \quad (2)$$

where s is determined with n degrees of freedom and B^2 is the term in the analysis of variance measuring the variability in y attributable to the slope b .

The values of λ in table 3 have been computed with Equations (1) and (2). In terms of λ , the three experiments were in agreement ($X^2 = 3.31$, $n = 2$, $P = 0.19$) and the weighted mean ($\bar{\lambda} = 0.0763 \pm .0068$) was but little greater than the standard deviation in terms of the log-dose directly ($s = 0.0644$, $n = 132$). However, the three experiments differed significantly in the slope of the dosage-response curve ($P = 0.024$), even though the ratio of the standard deviation to the slope varied within the sampling error.

THE CALCULATION OF RELATIVE POTENCIES. When the same sample of drug has been tested at four dosage levels spaced equally on a logarithmic scale, the

experiment can be analyzed alternatively as an assay. The first and third doses may be assumed to represent the standard, and the second and fourth doses an unknown preparation. A factorial analysis has been described (3) with which the terms essential for the estimation of potency can be computed from the totals of the responses to four such doses. The variances computed by this method are listed in the first three rows of table 4. The first row gives the variance attributable to the difference in potency between the standard and unknown, in every case it exceeded the error many times. The second row measures the average effect of the known difference between the two high doses and the two low doses

TABLE 4

Analysis as three assays in terms of log minutes survival, assuming that the first and third doses were the standard preparation and the second and fourth an unknown

	RESULTS FROM SEPARATE EXPERIMENTS				COMBINED	
	D F	I	II	III	D F	
Variance or mean square for						
Difference between samples (D^2)	1	06021	06109	08342	1	20445
Slope of parallel curves (B^2)	1	34037	48501	14997	1	92641
Divergence from parallelism	1	00480	00510	00005	1	00001
Error (s^2)	44	00364	00494	00493	132	00451
Calculation of relative potency						
Difference in mean response ($\bar{y}_S - \bar{y}_U$)		+ 07053	+ 07188	+ 08338		+ 07536
Slope of parallel curves (b)		- 86893	- 1 03726	- 57678		- 82766
Standard deviation in log dose (λ)		06048	06778	12180		09112
Potency from separate assays						
In logarithms $M \pm s_M$		0815	0693	1446		0911
		± 0218	± 0208	± 0439		± 0149
Comparison with true M (t)	44	707	1 329	1 086	132	392
Potency from combined b_s and λ_s						
In logarithms $M \pm s_M$		0856	0868	1007		0911
		± 0242	± 0242	± 0244		± 0149
In percentages		121 8	122 1	126 1		123 3
		$\pm 6 8$	$\pm 6 8$	$\pm 7 1$		$\pm 4 2$
Comparison with true M (t)	132	469	416	167	132	392

or that due to the slope of the best fitting pair of parallel lines for standard and unknown. It was by far the largest term. The third row tests the divergence from parallelism of separately fitted curves for standard and unknown. In no case was this so great as to throw doubt upon the use of parallel lines. These terms are evaluated by comparison with the mean square for error (s^2) in the fourth row of the table. It represents 44 degrees of freedom and includes the variation between groups and the interaction of groups with treatments.

The results for all three 'assays' have been combined in the last column of table 4. They are based on the differences between the totals of all three exper-

ments for each factor. In each row the combined value is less than the sum of the three variances for the individual experiments. The difference, with two degrees of freedom, is the sum of squares for the discrepancy between the three experiments in respect to each term. By this criterion there was good agreement in the first and third items but significant disagreement in slope, as would be anticipated from the preceding section.

Graded-response assays are generally computed as self-contained determinations of potency, and modern experimental designs have placed them upon a quantitative basis such as in reference (3). This would seem especially desirable here, in view of the significant discrepancy among the three experiments in slope. On the assumption that the mean log-doses were equal ($\bar{x}_s = \bar{x}_u$), the log-ratio of potency, M , and its error, s_M , have been computed for the separate "assays" by the equations

$$M = \frac{kID}{B} \quad (3)$$

and

$$s_M = s|M| \sqrt{\frac{1}{D^2} + \frac{1}{B^2}}, \quad (4)$$

where D , B and s are the square roots of the first, second and fourth entries in table 4, $|M|$ indicates that M is taken as positive, the log-interval between successive doses of both standard and unknown, $I = 2 \times 0.0969 = 0.1938$ and $k = 1$. These values were then compared with the known, true log-potency of 0.0969 by the t -test, none of the estimates diverging more than would be expected from their respective errors. The combined estimate of $M = 0.0199 \pm .0149$, computed with the combined slope $b_c = -0.8277$ approached the true value more closely than that for any individual experiment.

THE USE OF COMPOSITE VALUES IN COMPUTING POTENCY. When the same drug is assayed repeatedly in a given laboratory, its potency can be determined more efficiently through the effective use of past experience. Two statistics are determined repeatedly in each assay, the standard deviation of the response and the slope of the dosage-response curve. If these should vary only within the sampling error in successive experiments, values determined from a series of assays should lead to better estimates of relative potency and its error than if they were based upon a single experiment. The log-ratio of potencies may be computed as

$$M = \bar{x}_s - \bar{x}_u - \frac{\bar{y}_s - \bar{y}_u}{b_c} \quad (5)$$

where the mean log-doses, \bar{x}_s and \bar{x}_u , are in terms of full-strength tincture, the mean responses, \bar{y}_s and \bar{y}_u , are computed from the individual assay and the slope, b_c , is that determined from this and other similar, mutually consistent experiments. The standard error of M may then be written as

$$s_M = \lambda_c \sqrt{\frac{N_u + N_s}{N_u N_s} + \frac{(\bar{y}_u - \bar{y}_s)^2}{B^2}} \quad (6)$$

where N_U and N_S are the number of observations on standard and unknown respectively in the individual assay, λ_e as defined by Equation (1) is computed from pooled estimates of s and b_e , and B_e^2 represents the effect of slope in an analysis of variance for all experiments used in computing b_e . Thus, if

$$b_e = \frac{S[xy]}{S[x^2]}, \quad (7)$$

then

$$B_e^2 = \frac{S^2[xy]}{S[x^2]} = b_e S[xy], \quad (8)$$

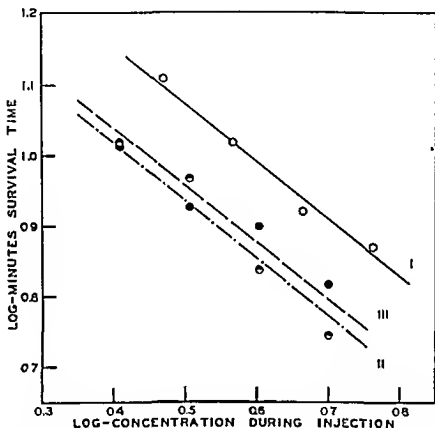


FIG. 2. DOSAGE-RESPONSE CURVES FOR DETERMINING THE RELATIVE POTENCIES OF THREE TINCTURES FROM THE LOGARITHM OF THE SURVIVAL TIME

where $S[xy]$ and $S[x^2]$ are, respectively, the sums of the numerators and of the denominators of the slopes for the individual experiments.

The use of the combined values, b_e and λ_e , in computing the relative potencies in the three experiments of table 4 has one serious objection. The odds were small ($P = 0.005$) that the individual slopes were samples from the same population. Against this finding was the consistency in $(\bar{y}_S - \bar{y}_U)$ and in s^2 . On the assumption that the differences in slope were the occasional rare large discrepancies which can arise by chance alone, the log-ratio of potency and its error have been recomputed by Equations (5) and (6) for each experiment, taking the values for B_e^2 , b_e and λ_e from the last column of table 4 and the differences

$(\bar{y}_U - \bar{y}_S)$ from the columns for the individual experiments. In each case, of course, $N_U = N_S = 24$. The resulting potencies have been listed in the last rows of table 4 and compared by the statistic t with the true value of $M = 0.0969$ or 125 per cent potency. In every case the new assayed potency was nearer the true value than when computed individually.

THE RELATIVE POTENCY OF THE THREE PREPARATIONS OF DIGITALIS. The log-survival time provides a method for measuring the relative potency of the three preparations used in the present experiments. The mean response at each log-dose has been plotted for each sample in figure 2. Parallel lines with the combined slope, $b_c = -0.8177$ were then drawn through the mean response for each experiment at its mean log-dose (table 3).

Designating the most potent of the three preparations as the standard, that in Experiment II, the log-ratio of potencies for the other two have been computed by Equations (5) and (6) as $M_I = -0.1679 \pm .0181$ and $M_{III} = -0.0270 \pm .0168$. The variance of the plotted mean values about the three parallel lines in figure 2 was not significantly larger than that of the individual y 's about their respective means ($F = 1.68, n_1 = 8, n_2 = 132, P > 0.1$). In view of this finding and the greater accuracy obtained with a combined slope in the analysis as test assays, the three preparations have been handled here as if any lack of parallelism in their dosage-response curves were fortuitous. Assigning the sample of digitalis in the second experiment a potency of 100 per cent, that of the first experiment has a potency of 67.9 ± 2.8 per cent and that of the third experiment a potency of 94.0 ± 3.6 per cent.

SUMMARY AND CONCLUSIONS

In the assay for digitalis the U.S.P. XII calls for adjusting the concentration of the injection fluid so that it will contain the estimated fatal dose per kg. in 15 cc. This is injected at the rate of one cc./kg. every 5 minutes and the average number of injections in an acceptable group of cats must not exceed limits of 13 to 19 for an official assay. To test the importance of this restriction four groups each of 12 cats have been injected with dilutions containing 64, 80, 100 and 125 per cent respectively of the estimated fatal dose, in each case at the rate of one cc. per kg. The experiment has been made with three different preparations of digitalis, requiring a total of 144 cats.

In three experiments the lethal dose increased with the concentration of digitalis in the injection fluid which, in turn, controlled the rate of injection. The increase was statistically significant in 2 of the 3 cases. The relation between log-dose and log-concentration was linear and has been fitted by a straight line with an average slope of $b = 0.20 \pm 0.05$. In other words, a two-fold increase in the concentration of tincture in the injection fluid increased the lethal dose in terms of the original tincture by approximately 15 per cent, apart from any other change in procedure. If the groups of cats comprising an assay were to require 13 and 19 injections respectively, the greatest allowable difference, the percentage potency of the sample could be biased by 8 per cent through this factor alone. Since the relation between log-concentration and log-dose in these experiments is

linear, the required concentration of the dilute solution could be decreased to the present minimum and yet not eliminate this factor. The potential bias could be reduced, however, by restricting the allowable difference in the average number of injections between the two groups of a single assay.

An alternative procedure for the cat assay has been examined with the present data, based upon the linear relation between log concentration of the injection fluid and the log minutes survival time. In design and analysis it is equivalent to an assay based upon a graded response. The methods of statistical analysis suitable for self contained assays have been extended, so that past experience can be used as it accumulates. When the present experiments were computed as test assays by this procedure, the estimated potencies agreed very well with the true value. The main advantage of the new technique is that it does not require as accurate a forecast of the potency of an unknown preparation as the official assay, its main disadvantage is that it would not reduce the number of cats needed to obtain a given precision. It should be of value, however, in the preliminary experiments which usually precede an official assay.

We are indebted for technical assistance to Mr. Walter Bachinski and to Miss Barbara Bartels. This study has been aided by a grant from the Gustavus and Louise Pfeiffer Research Foundation.

REFERENCES

- 1 BLISS, C. I. The U. S. P. collaborative cat assays for digitalis. *J. Am. Pharm. Ass'n* 33, 225, 1944.
- 2 BLISS, C. I., AND HANSON, J. C., *J. Am. Pharm. Ass'n*, 28, 521, 1939.
- 3 BLISS, C. I., AND MARKS, H. P., *Quart. J. Pharm. and Pharmacol.*, 12, 82-182, 1939.
- 4 VOS, B. J. AND DAWSON, W. T., *J. Pharmacol. and Exptl. Ther.*, 57, 145, 1936.

STREPTOTHRICIN AS A CHEMOTHERAPEUTIC AGENT

HARRY J. ROBINSON AND DOROTHY G. SMITH

Received for publication June 7, 1944

During the past fifteen years three discoveries were made which have had a marked influence on the course of bacterial chemotherapy and related fields. The first discovery made by Fleming (1) in 1929 was the forerunner of the work on penicillin by Chain, Florey et al (2); the second made by Domagk (3) in 1935 led to the synthesis of numerous sulfonamides; and the third, made by Dubos in 1939 (4), stimulated intensive search for chemotherapeutic agents among the microbial population of the water and soil. All approaches proved highly successful, as evidenced by the appearance of a number of useful chemotherapeutic agents, such as sulfanilamide, sulfadiazine, tyrothricin and penicillin.

The majority of the preparations introduced to date appear to be mainly effective in infections produced by gram-positive bacteria, although a few gram-negative species, such as the gonococcus and meningococcus are extremely sensitive to the sulfonamides (5, 6), as well as to certain antibiotic agents (7, 8). Reports also suggest that while a few sulfonamides (9, 10) appear to be useful in the treatment of bacillary dysentery, the occurrence of toxic effects, such as urolithiasis (11), nausea and vomiting (12) leaves much to be desired in the chemotherapy of this disease. Most gram-negative bacterial infections, however, such as those related to the Colon-typhoid, Salmonella and Brucella groups, remain quite refractory to treatment with the chemotherapeutic agents available to date. In view of these facts, as well as the apparently increased incidence of primary and secondary gram-negative bacterial infections resulting from the war, the availability of an agent capable of curing local and systemic gram-negative infections in man and animals would seem of particular importance at this time.

In 1940 Waksman and Woodruff (13) undertook a study designed to obtain micro-organisms antagonistic to gram-negative bacteria. In the course of their investigation, several antagonistic actinomycetes were isolated and studied. Among those of particular interest was an organism named *Actinomyces laven-dulae*, which produced under certain conditions a new antibiotic agent named streptothricin. In contrast to most chemotherapeutic agents of microbial origin, such as penicillin (14) and gramicidin (15), whose action is directed primarily towards the gram-positive bacterial forms, streptothricin proved highly active *in vitro* against all gram-negative bacteria tested. A recent publication by Foster and Woodruff (16) showed streptothricin to be active also *in vitro* against a number of gram-positive bacteria and fungi. A preliminary study by Metzger et al (17) suggested that crude streptothricin was effective in controlling the course of Brucella infections in guinea pigs, while one of us (H. R.) found the drug active *in vivo* against *E. coli* in mice (18).

The present communication is primarily concerned with the efficacy of crude

streptothricin in a variety of bacterial infections, and also in infections produced by *Trypanosoma equiperdum* and the virus of epidemic influenza

MATERIALS *Streptothricin*¹ The crude streptothricin used in this study varied in potency from 5,000 to 300,000 units² per gram, and was isolated from two types of culture media described by Waksman and Woodruff (13) and Woodruff and Foster (19) Although there was considerable difference in the toxicological and pharmacological properties of the streptothricin obtained from the two types of media, there appeared to be little difference in their behavior towards bacteria *in vitro* or *in vivo* as determined on a unit basis

Animals Mice of the Carworth Farms, CFI strain, weighing about 18-22 grams each were used for the bacterial *in vivo* tests The CFW strain, weighing 9-11 grams, was employed in the virus studies The animals were maintained on a balanced diet, with water available at all times

Bacterial Strains A variety of pathogenic gram-positive and gram-negative cocci and bacilli were employed in the *in vitro* and *in vivo* studies Most strains were highly mouse virulent, and were maintained at peak virulence by frequent passage through mice In most cases the organisms were grown at 37°C for six hours in brain-heart infusion media In the case of certain fastidious organisms, such as *Strep hemolyticus* and *Diplo pneumoniae*, the brain heart medium was supplemented with 10% defibrinated rabbit blood, and for growth of the strict anaerobes, with 0.1% agar agar

The virus and protozoan studies were performed using the virus of epidemic influenza, strain PR-8, and a strain of *Trypanosoma equiperdum* In each case, the culture was maintained by serial passage through mice

Methods *In vitro* Studies Three testing procedures were used for determining the bacteriostatic and bactericidal activity of streptothricin against pathogenic bacteria These have been described in previous communications (20-21), and therefore will only be referred to here as Kolmer's method, the agar plate method, and the rotating rack testing procedure

The *in vitro* assay performed with the influenza virus consisted of mixing the infected lung suspension with the drug on a mixing machine which rotated slowly at room temperature throughout a 24 hour test period The viability of the virus was then determined by diluting the mixture in a 50% serum-saline solution through 10^{-4} and inoculating 0.02 cc of the 10^{-4} , 10^{-5} and 10^{-6} dilutions intranasally into mice of the CFW strain Control experiments were performed at the same time by mixing the lung suspension with sterile saline in order to be certain that the virus remained viable and infective under these conditions

In vivo Studies—For the bacteriological tests, six hour broth cultures of the test organism were diluted in broth or 4% mucin through 10^{-2} , and 0.5 cc of the 10^{-4} , 10^{-5} and 10^{-6} culture dilution was injected intraperitoneally into each of the test mice Titration tests and blood agar pour plates showed this quantity of culture to vary between 10,000 and 100,000 lethal doses of bacteria depending on the particular strain employed Treatment with streptothricin was initiated at various time intervals following the bacterial inocula-

¹ The streptothricin employed in these studies was obtained from Drs R Denkwalter, R Peck, M Tishler and A Waltz of the Research Laboratories of Merck & Co Inc, from cultures grown by Dr J W Foster

² A unit of streptothricin is the minimum quantity of drug which when added to 1.0 cc of nutrient broth will inhibit a given strain of *E. coli*

tion, and ranged from one minute to 10 hours after the injection of the test organism. The drug was given by intravenous, subcutaneous, intraperitoneal and oral administration in varying dose levels and at varying time intervals after the bacterial infection.

For the *in vivo* experiments with the influenza virus, a lung suspension was diluted through 10^{-6} in the serum-saline solution, and 0.02 cc. of the 10^{-4} , 10^{-5} and 10^{-6} dilution was inoculated intranasally into mice. Treatment by subcutaneous injection was initiated immediately following the intranasal inoculation, and repeated once daily or at 6 hour intervals until death of the animal.

In the case of the *Trypanosoma equiperdum* infections, the blood of an infected mouse was diluted in citrated saline so that two to three organisms were found per field (440X). One-half cc. of this blood dilution was inoculated intraperitoneally into each test mouse. Treatment was the same as for the virus mice.

Throughout the course of the bacterial *in vitro* and *in vivo* studies, a number of observations were made on the morphology of the test organisms. The effect of streptothricin in certain cellular and humoral defense mechanisms of the host, such as phagocytosis and agglutinin production were also studied. The action of the drug on bacterial toxins and virulence was considered.

TABLE 1
Acute toxicity of streptothricin for mice

DOSE	NO. OF MICE/DOSE	PER CENT MORTALITY 5 DAY OBSERVATION		
		I.V.	S.C.	Oral
<i>units/kgm.</i>				
30,000	10	0	0	0
60,000	10	20	0	0
125,000	10	20	30	0
250,000	10	80	100	0
500,000	10	100	100	10
750,000	10	100	100	30

Toxicity. The results concerned with the toxicity and pharmacological properties of streptothricin will be reported elsewhere (22). However, a summary of the acute toxicity in mice of the streptothricin employed in these studies is given in Table 1, in order to evaluate the chemotherapeutic index of this material.

RESULTS OF BACTERIOLOGICAL TESTS IN VITRO

Agar Plate Method. Table 2 summarizes the results of these experiments. Streptothricin in relatively small amounts was bacteriostatic to a variety of gram-positive and gram-negative pathogenic bacteria. The drug was particularly effective against gram-negative bacteria, although certain species, such as *B. pyocyaneus* and *B. proteus* showed considerable resistance. Thus, while concentrations of 4 units per cc. were required to completely inhibit the growth of *E. typhi*, quantities as great as 512 units per cc. had no apparent effect on *B. proteus*. There also appeared to be a considerable difference in the resistance of strains among the same species, as evidenced by the results obtained with four strains of *Strep. hemolyticus* and four strains of *Staph. aureus*. *Strep. viridans* and *Strep. lactis* were not influenced by concentrations of streptothricin as great as 1024 units per cc. Both organisms were previously found highly resistant to penicillin (21).

Kolmer's Method The results of the bacteriostatic test obtained with this testing method were in many respects similar to those described above. In addition, however, it was possible to demonstrate by this procedure that streptothricin exerted a killing effect as well as an inhibitive action upon the same organisms shown in table 2. Streptothricin was relatively ineffective against the

TABLE 2
Bacteriostatic action of streptothricin agar plate method

ORGANISM	CONCENTRATION REQUIRED TO PRODUCE COMPLETE INHIBITION
	<i>streptothricin units per cc of agar</i>
<i>Strep hemolyticus</i> 1685	32
<i>Strep hemolyticus</i> MIT	256
<i>Strep hemolyticus</i> M	256
<i>Strep viridans</i>	1024
<i>Strep lactis</i>	1024
<i>Staph aureus</i> SM	16
<i>Staph aureus</i> FDA	128
<i>Staph aureus</i> SD	128
<i>Staph aureus</i> 155	128
<i>Diplo pneumoniae</i> Type I	32
<i>B mycoides</i>	1024
<i>B subtilis</i>	32
<i>E typhi</i>	4
<i>S aertrycke</i>	16
<i>S enteritidis</i>	64
<i>S schottmülleri</i>	16
<i>B flezneri</i>	32
<i>B sonne</i>	128
<i>P lepiaseptica</i>	32
<i>B proteus</i>	512
<i>B pyocyaneus</i>	256
<i>N meningitidis</i>	256
<i>E coli</i>	16
<i>S leutea</i>	256
<i>A aerogenes</i>	256
KOLMER'S METHOD	
<i>Cl welchii</i>	>855
<i>Cl tetani</i>	>1080
<i>Cl sordelli</i>	>1080
<i>Cl septicæ</i>	540
<i>Cl novyi</i>	270

gram positive anaerobic pathogens, such as *Cl tetani*, *Cl welchii*, *Cl septicæ* and *Cl sordelli*, in that these organisms were not inhibited at the maximum concentration of streptothricin employed. The drug had some killing effect on *Cl novyi*.

Rotating Rack Method The results of a typical experiment are given in

table 3 and show that quantities as small as 5 units of streptothricin per cubic centimeter of broth exert a killing effect upon *S. schottmülleri* within 2 to 4 hours. Smaller amounts of 1.25 and 2.5 units retarded the growth of the test organism over a 10-hour period, but eventually the organisms multiply and become abundant. Similar results were obtained with *E. coli*, *S. aertrycke*, *E. typhi* and *Staph. aureus* (Smith). Other strains of hemolytic streptococci and pneumococci were somewhat more resistant. When blood was used as the test medium, the results were modified somewhat, but such changes were explainable solely on the basis of the stimulating, or in some cases, the inhibiting effect fresh rabbit blood had on the test organism.

Factors Influencing the Activity of Streptothricin In Vitro. Foster and Woodruff (16) showed that the pH of the assay medium and the presence of inorganic salts influenced the *in vitro* activity of streptothricin, while the size of the inocu-

TABLE 3
In vitro efficacy of streptothricin in broth against *S. schottmülleri* (rotating rack technique)

TUBE NO.	STREPTOTHRICIN PER CC. OF BROTH UNITS	NUMBER OF VIABLE BACTERIA/0.1 CC. OF BROTH						
		Time in hours						
		0	2	4	6	8	10	24
1	0	1750	∞	∞	∞	∞	∞	∞
2	0	2000	∞	∞	∞	∞	∞	∞
3	0.62	2100	∞	∞	40	11	10	∞
4	1.25	1800	3000	41	1	10	8	∞
5	2.5	1850	480	14	57	500	∞	∞
6	5.0	2100	4	0	0	0	0	0
7	10.0	1920	0	0	0	0	0	0
8	20.0	1850	0	0	0	0	0	0

∞ = Infinity.

lum was of no particular significance. Our experiments confirm these findings and show further that blood, serum, peptone and a number of vitamins of the B Complex, including thiamin, riboflavin, pantothenic acid and pyridoxine have no significant influence on the activity of streptothricin. Since the experiments were performed with *E. coli* grown in a synthetic medium (23), the slight inhibitory effect of certain of the foregoing substances was attributed to the stimulating effect they had on the growth of the test organism.

Morphological Effects. Similar to the findings of Foster and Woodruff (16), streptothricin was found to produce marked morphological changes resembling those reported in the case of penicillin (24) and the sulfonamides (25). These consisted of marked elongation in the case of bacilli, and a considerable increase in diameter with cocci, resulting in the production of many bizarre forms. The staining characteristics were also modified somewhat in that gram-positive organisms frequently appeared to be gram-negative.

RESULTS OF IN VIVO TESTS

Intravenous Therapy Single doses of 100 units of streptothricin (0.33 mgm/0.03 cc unit material) given by intravenous injection within ten to fifteen minutes after the bacterial inoculation protected 94% of the mice infected with 100–1000 µl doses of *S. schottmülleri*, as shown in table 4. Smaller amounts of the drug offered some protection, as evidenced by the survival of 56% of the mice on

TABLE 4

Efficacy of streptothricin in mice infected with S. schottmülleri (Intravenous therapy)

anism of culture ction	<i>Solmonella schottmüllers</i> 6 hours 0.5 cc. of a 10 ⁻⁵ culture dilution in 4% mucin injected intra peritoneally Streptothricin given intravenously immediately after bac terial inoculation												
atment													
OF E	DRUG	UNITS/ DOSE	NO OF DOSES/ DAY	CULTURE DILUTION	NO SURVIVING IN DAYS								% SUR VIVAL
					1	2	3	4	5	6	7	8	
Therapy a single dose													
	Strepto thricin	3 1	1	10 ⁻⁵	7	6	5	5	4	4	3	3	7 5
		6 2	1	10 ⁻⁵	5	4	3	3	3	3	3	3	7 5
		12 0	1	10 ⁻⁵	5	2	1	1	1	1	1	1	2 5
		25 0	1	10 ⁻⁵	16	10	10	8	7	7	7	6	12 0
		50 0	1	10 ⁻⁵	43	33	32	31	29	29	20	28	56 0
		100 0	1	10 ⁻⁵	50	40	49	48	47	47	47	47	04 0
Therapy single daily doses over a 3 day period													
	Strepto- thricin	50	1	10 ⁻⁵	10	10	10	10	10	10	10	10	100
		100	1	10 ⁻⁵	10	10	10	10	10	10	10	10	100
		200	1	10 ⁻⁵	10	10	10	10	10	10	10	10	100
Therapy none													
	Controls			10 ⁻⁵	1	0	0	0	0	0	0	0	0
				10 ⁻⁴	13	0	0	0	0	0	0	0	0
				10 ⁻³	15	7	6	6	5	5	5	5	20
				10 ⁻²	18	12	9	9	7	6	5	4	16

50 unit dose level and 12% on the 25 unit dose. Repeated doses of 50, or 200 units per mouse, given once daily over a three day period protected the mice.

Intraperitoneal Therapy When both test organism and the drug were given intraperitoneal injection, concentrations as small as 12.5 units per mouse were sufficient to protect 77% of the mice infected with *S. schottmülleri* (table 5). Treatment was usually

the bacterial injection, although essentially the same results were obtained if treatment was delayed for 30 to 45 minutes. The effective dose under these conditions was similar to that required for effecting a bactericidal action *in vitro* and the results suggest, therefore, that the mechanism of action *in vitro* and *in vivo* is essentially the same.

Subcutaneous Therapy. Streptothricin in a single dose of from 100 to 200 units per mouse (5000 to 10,000 units/kgm.) given by the subcutaneous route immediately after the bacterial inoculation protected mice against a variety of gram-negative bacterial infections. Since the results were similar for a number of bacterial species, only the results with one test organism are given in table 6.

TABLE 5

Efficacy of streptothricin in mice infected with S. schottmülleri (Intraperitoneal therapy)

Organism.....	<i>Salmonella schottmülleri</i>
Age of Culture.....	6 hours
Infection.....	0.5 cc. of a 10^{-8} culture dilution in 4% mucin injected intraperitoneally.
Treatment.....	Streptothricin given intraperitoneally immediately after bacterial inoculation

NO. OF MICE	DRUG	UNITS/ DOSE	NO. OF DOSES/ DAY	CULTURE DILUTION	NO. SURVIVING IN DAYS								SURVIVAL
					1	2	3	4	5	6	7	8	
Therapy: a single dose													
40	Strepto- thricin	3.1	1	10^{-5}	5	4	4	3	3	3	3	3	7.5
40		6.2	1	10^{-5}	19	18	17	13	12	12	12	12	30.0
40		12.5	1	10^{-6}	36	31	31	31	31	31	31	31	77.5
40		25.0	1	10^{-6}	35	33	33	33	33	33	33	33	82.5
40		50.0	1	10^{-6}	37	37	37	37	37	37	37	37	92.5
Therapy: none													
40	Controls			10^{-5}	3	1	0	0	0	0	0	0	0
20				10^{-6}	0	0	0	0	0	0	0	0	0
20				10^{-7}	7	6	0	0	0	0	0	0	0
20				10^{-8}	7	4	3	2	2	2	2	2	10

Among the bacteria which are particularly sensitive to streptothricin are *E. coli*, *S. schottmülleri*, *S. aertrycke* and *E. typhi*. Quantities less than 100 units also afforded some protection in the case of the foregoing organisms, in that doses of 50 units per mouse protected 50% of the animals. The lives of mice infected with *B. proteus* and *B. pyocyaneus* were prolonged somewhat by streptothricin therapy, but eventually most of the animals died.

When streptothricin was administered once daily over a five-day period, the results were essentially the same as those obtained by a single treatment. However, when the drug was given repeatedly every six hours over a five-day period, the effectiveness of streptothricin was enhanced to the extent that 50 units per

mouse protected 95% of the animals (table 6) These results suggest that streptothricin, like penicillin, is rapidly excreted or destroyed in the body Preliminary findings show, however, that a large proportion of the drug is excreted by the kidney (26)

TABLE 6

Efficacy of streptothricin in mice infected with S. schottmülleri (Subcutaneous therapy)

Organism	<i>Salmonella schottmülleri</i>												
Age of culture	6 hours												
Infection	0.5 cc of a 10 ⁻⁶ culture dilution in 4% mucin												
Therapy	Streptothricin given subcutaneously immediately after bacterial inoculation												
NO OF MICE	DRUG	UNITS/DOSE	NO OF DOSES/DAY	CULTURE DILUTION	NO SURVIVING IN DAYS								% SURVIVAL
					1	2	3	4	5	6	7	8	
Therapy a single dose													
30	Streptothricin	12.5	1	10 ⁻⁴	3	0	0	0	0	0	0	0	0
65		25.0	1	10 ⁻⁴	25	23	18	15	15	15	14	21.5	
65		50.0	1	10 ⁻⁴	49	44	43	41	37	37	34	34	52.4
65		100.0	1	10 ⁻⁴	65	62	52	60	59	59	59	59	90.8
35		200.0	1	10 ⁻⁴	35	35	35	35	35	35	35	35	100.0
Therapy single daily doses over a 5 day period													
20	Streptothricin	12.5	1	10 ⁻⁴	2	1	0	0	0	0	0	0	0
20		25.0	1	10 ⁻⁴	9	5	4	3	1	1	1	1	5
20		50.0	1	10 ⁻⁴	18	13	12	12	12	11	11	11	55
Therapy every 6 hours over a 5 day period													
20	Streptothricin	12.5	4	10 ⁻⁴	20	4	3	3	3	3	3	3	15
20		25.0	4	10 ⁻⁴	20	6	3	3	1	1	1	1	5
20		50.0	4	10 ⁻⁴	20	20	20	20	20	20	20	19	95
20		100.0	4	10 ⁻⁴	20	20	20	20	20	20	20	20	100
Therapy none													
65	Controls			10 ⁻⁴	0	1	0	0	0	0	0	0	0
30				10 ⁻⁶	3	0	0	0	0	0	0	0	0
30				10 ⁻⁷	15	10	7	5	3	3	3	3	10
30				10 ⁻⁸	10	9	8	7	6	0	6	0	20

Of the gram-positive bacterial infections studied, single doses of streptothricin afforded protection to mice infected with *Strep. hemolyticus* 1685 when administered subcutaneously in doses of 1600 units per 20 gram mouse. On the other hand, the drug was not very active against strains of *Diplo. pneumoniae*, or *Staph. aureus*, regardless of the dose administered. However, the amount of streptothricin administered to the foregoing mice approached the toxic level, and a number of deaths were due to the drug rather than the infection.

Oral Administration. Streptothricin was considerably less effective when given by mouth than when administered parenterally. However, if sufficiently large doses were fed, mice were protected against a heavy infection produced by *S. schottmülleri*. Doses of 1500 units/20 gm. mouse given as a single dose shortly after the intraperitoneal injection of the bacteria, were sufficient to protect 50% of the mice (table 7). Doses smaller than this offered some protection, but a large percentage of the mice eventually died. Sufficient quantities of the material were not available to study other infections under these conditions, but all other *in vitro* and *in vivo* findings suggest that the drug will also be effective against other gram-negative bacteria when given by mouth. It will be recalled

TABLE 7

Efficacy of streptothricin in mice infected with S. schottmülleri (Oral administration)

Organism.....	Salmonella schottmülleri
Age of Culture.....	6 hours
Infection.....	0.5 cc. of a 10^{-5} culture dilution in 4% mucin
Therapy.....	Streptothricin given orally immediately after bacterial inoculation.

NO. OF MICE	DRUG	UNITS/ DOSE	NO. OF DOSES/ DAY	CULTURE DILUTION	NO. SURVIVING IN DAYS								% SUR- VIVAL
					1	2	3	4	5	6	7	8	
Therapy: a single dose													
20	Strepto- thricin	93.75	1	10^{-5}	1	1	0	0	0	0	0	0	0
20		187.5	1	10^{-5}	6	1	1	1	1	1	1	1	5
50		375.0	1	10^{-5}	20	13	12	11	9	9	9	9	18
50		750.0	1	10^{-5}	30	27	23	22	22	22	19	19	38
50		1500.0	1	10^{-5}	34	27	27	27	26	25	25	25	50
50	3000.0	1	10^{-5}	48	48	46	44	42	38	38	38	76	
Therapy: none													
40	Controls			10^{-5}	1	1	0	0	0	0	0	0	0
15				10^{-6}	0	0	0	0	0	0	0	0	0
15				10^{-7}	5	2	1	1	1	1	1	0	0
15				10^{-8}	7	6	5	4	4	4	4	4	26

that streptothricin is considerably less toxic when given by mouth than by parenteral administration, and therefore the chemotherapeutic index is not necessarily less than that obtained following parenteral therapy.

Influence of the size of the bacterial inoculum and the time interval between infection and treatment on streptothricin activity. Doses of 100 to 200 units of streptothricin afforded excellent protection to mice even when administered to animals infected with 100,000 to 1,000,000 lethal doses of *S. schottmülleri*. The results suggest, as did the *in vitro* findings, that the activity of streptothricin is not markedly influenced by the presence of large numbers of bacteria. Likewise, when mice were infected with 1000 lethal doses of the above test organism, and therapy

delayed for 1, 4, 8 and 10 hours, streptothricin protected all the animals from death, provided sufficiently large doses of the drug were given.

RESULTS OF VIRUS AND PROTOZOAN STUDIES Streptothricin showed little or no activity against the virus of epidemic influenza, even when quantities of the drug approaching the toxic dose range were used. Likewise, the drug was unable to protect any of the mice infected with *Trypanosoma equiperdum*, although the progress of the infection was retarded slightly.

Mechanism of Action All the evidence available on the mode of action of the sulfonamides suggests that these drugs act directly on the bacteria, producing a bacteriostatic effect which allows the defense mechanisms of the body to cope with the infection. The data presented here suggest that in contrast to the sulfonamides, streptothricin in sufficiently large doses produces a bactericidal effect *in vivo*, as well as *in vitro*, thus reducing considerably the action required on the part of the host.

In order to determine the rate at which bacteria were killed in the peritoneal cavity of mice following streptothricin therapy, a series of experiments was performed in which mice were infected by intraperitoneal injection and treated subcutaneously or intraperitoneally with therapeutic doses of streptothricin. The mice were sacrificed at hourly intervals and the number of viable bacteria present in the peritoneal cavity determined by flushing out the cavity with 0.5 cc. of saline and plating this fluid in nutrient agar. The plates were incubated at 37°C. and counts made of the number of colonies.

Similar experiments were performed with infected mice that were not treated with streptothricin. Finally, it was realized that small amounts of streptothricin present in the peritoneal washings would continue to exert a bactericidal effect after the fluid was added to the nutrient agar. In order to eliminate this artifact, the amount of streptothricin present in the peritoneal fluid was determined by a modification of the agar cup method described by Foster and Woodruff (27).

Under the conditions of these experiments, streptothricin was found to sterilize the peritoneal cavity within 4-6 hours after the bacterial inoculation. Simultaneously with the killing effect, there was an increased incidence of phagocytosis, as determined by examination of the stained peritoneal fluid. From the *in vitro* results presented in table 3, one may see that streptothricin kills a culture of *S. schottmulleri* within the same time interval, further illustrating the similarity between the *in vitro* and *in vivo* action.

The question as to whether streptothricin has any influence on antibody formation was determined in mice by examining the serum of infected mice treated with varying doses of streptothricin. The results show that if the dose of streptothricin is sufficiently small, so that the mice show signs of the infection, the serum of such animals contains agglutinins for the infecting bacteria. However, with larger doses of the drug, none of the animals became sick, and no agglutinins were found in the serum. Animals which developed agglutinins in this manner showed considerable resistance to re-infection, in that all such mice survived an infecting dose of bacteria which killed all the normal controls. It

is possible that a practical method of animal immunization can be established by this procedure.

As far as could be determined, streptothricin had no influence on the toxins of *Cl. welchii* or *Cl. tetani*, suggesting that the factor of toxin inactivation plays no role in the action of streptothricin.

Discussion. The properties of crude streptothricin as described in this report suggest that this agent offers many possibilities as a chemotherapeutic agent useful in the treatment of bacterial infections produced by gram-negative pathogens. Organisms of the *Colon-typhoid* and *Salmonella* groups appear to be particularly sensitive to the action of streptothricin, while others such as *B. pyocyaneus* and *B. proteus* show considerable resistance.

Even the more resistant gram-negative organisms, as well as a number of gram-positive organisms are inhibited *in vitro* by streptothricin. In view of the close correlation between the *in vitro* and *in vivo* results, one might expect streptothricin to be effective *in vivo* against these resistant forms, providing sufficient amounts of the drug can be administered. In the last analysis, therefore, the ultimate value of streptothricin will depend largely on the toxicity of the purified product. The results available to date, even with the crude product, indicate that a satisfactory ratio of 8 to 10-fold exists between the curative and acute toxic dose of streptothricin in infections produced by gram-negative *Colon-typhoid* or *Salmonella* groups.

The marked influence of streptothricin *in vitro* against a large variety of gram-positive and gram-negative bacteria, coupled with the fact that the drug is not inhibited by the presence of body fluids such as blood and serum, suggests that this agent will be of definite value even in its present crude form, in the treatment of infected burns and wounds or in preventing such infections.

The reduction in activity and toxicity of streptothricin when given by mouth suggests that the drug is either destroyed in the gastro-intestinal tract, or that it is not fully absorbed. Studies concerned with the nature of this phenomenon are now in progress. Preliminary studies indicate, however, that effective blood concentrations of the drug are obtained following oral administration, and that the drug also appears in the urine. The marked stability shown by streptothricin in the presence of strong acids suggests that, unlike penicillin, the gastric acidity probably has no influence on the reduced effect of streptothricin following oral administration.

As previously indicated, however, streptothricin per orally has a marked influence on the lactose-fermenting bacteria of the intestinal tract, and therefore may be of definite value in the treatment of bacillary dysentery and typhoid fever. In addition, food poisoning produced by organisms of the *Salmonella* group might be expected to respond readily to streptothricin therapy.

The value of streptothricin as a chemotherapeutic agent in the foregoing infections will depend largely upon the nature of the toxic effects produced by single and repeated doses of the drug. Much needs to be done in this direction.

SUMMARY

1. *In vitro* and *in vivo* studies show that crude streptothricin possesses marked activity against a variety of gram-negative bacterial species. Gram-positive forms are also sensitive to the action of streptothricin, but not to the same degree as the gram-negative form.

2. The activity of streptothricin is not influenced by blood, serum, peptone, or vitamins of the B complex.

3. Streptothricin is more active when given parenterally than when administered by mouth.

4. The drug is not active against the virus of epidemic influenza or *Trypanosoma equiperdum*.

5. The ultimate chemotherapeutic index of streptothricin will depend upon the toxicity of the pure product.

6. The results suggest that streptothricin may be useful in the local treatment of infected wounds and burns, as well as bacillary dysentery, typhoid fever and food poisoning produced by the *Salmonella* organisms.

REFERENCES

- (1) FLEMING, A, *Brit J Exp Path*, 10: 226, 1929
- (2) CHAIN, FLOREY, GARDNER, HEATLEY, JANNINOS, ORR-EWING, AND SANDERS, *Lancet*, 2: 226, 1940
- (3) DOMAG, G, *Deutsche Med Wchnschr*, 61: 250, 1935
- (4) DUBOS, J, *J Exp Med*, 70: 249, 1939
- (5) MARONEY, J F, C J VAN SLYKE, AND R R WOLCOTT, *Ven Dis. Inf*, 22: 425, 1941.
- (6) LEPPER, M H, L K SWIFT, AND H F DOWLING, *J A M A*, 123: 134, 1943
- (7) HERRELL, W E, E N COOK, AND L THOMPSON, *J A M A*, 122: 269, 1943
- (8) RAMMELKAMP, C H AND C S KLEFER, *Am J M Sc*, 205: 342, 1943
- (9) CRINO, R E, O S WARR, JR, AND J B WITHERINGTON, *J Tenn M A*, 34: 171, 1941
- (10) MARSHALL, E K, JR, *Miss Doctor*, 20: 4, 1942
- (11) LINDNER, H J, AND D W ATCHESON, *J Urol*, 47: 262, 1942
- (12) LOVO, P H, J W HAWLAND, L B EDWARDS, AND E A BLISS, *J A M A*, 115: 364, 1940
- (13) WAKSMAN, S A, AND H B WOODRUFF, *J Bact*, 40: 531, 1940
- (14) DAWSON, M H, G L HOBBY, K MYER, AND E CHAFFEE, *J Clin. Invest*, 20: 434, 1941
- (15) DUBOS, J, *J Exp Med*, 70: 1, 1939
- (16) FOSTER, J W, AND H B WOODRUFF, *Arch Biochem*, 3: 241, 1943
- (17) MELTZER, H J, S A WAKSMAN, AND L H PUOH, *Proc Soc Exp Biol and Med*, 51: 251, 1942
- (18) ROBINSON, H J, Thesis
- (19) WOODRUFF, H B, AND J W FOSTER, *Arch Biochem*, 2: 301, 1943
- (20) ROBINSON, H J, AND O E GRAESSLE, *This Journal*, 76: 316, 1942
- (21) ROBINSON, H J, *This Journal*, 77: 70, 1943
- (22) ROBINSON, H J, AND O E GRAESSLE, Unpublished data
- (23) KOHN, H I, AND J S HARRIS, *This Journal*, 73: 344, 1941
- (24) SMITH, L D, AND T HAY, *J Franklin Inst*, 233: 593, 1942.
- (25) LOCKWOOD, J S, *J Immun*, 35: 155, 1935
- (26) ROBINSON, H J, AND R B STEBBINS, Unpublished data
- (27) FOSTER, J W, AND H B WOODRUFF, *J Bact*, 47: 43, 1944

COMPARATIVE ANTICONVULSIVE ACTION OF
3,5,5-TRIMETHYLOXAZOLIDINE-2,4-DIONE
(TRIDIONE), DILANTIN AND PHENOBARBITAL

GUY M. EVERETT AND RICHARD K. RICHARDS

Department of Pharmacology, Abbott Laboratories, North Chicago, Illinois

Received for publication June 12, 1944

Hypnotic properties of oxazolidine-2,4-dione derivatives were first described by Erlenmeyer (1). Recently Luton, Blalock, Baxter and Stoughton (2) studied a series of 5,5-dialkyloxazolidine-2,4-diones and concluded that the di-n-propyl derivatives (Propazone) was the most promising as a hypnotic and also reported its use in a small group of epileptics. We have investigated N-methylated 5-alkyl and 5,5-dialkyloxazolidine-2,4-diones synthesized by Dr. M. A. Spielman (3) of the Abbott Laboratories. In this series the hypnotic action is greatly reduced when the alkyl radicals are small, and a definite analgesic effect becomes apparent (4). The present report concerns the marked anticonvulsive properties of 3,5,5-trimethyloxazolidine-2,4-dione (Tridione) which was also found to be the most potent analgesic in this group of compounds.

METHODS AND MATERIALS. Most of the convulsant drug studies were made on mice. In a few experiments, guinea pigs, cats and rabbits were used. The electrically induced seizures were studied in rats using a special helmet electrode which was fixed rigidly to the shaved head. The second electrode was held firmly to the roof of the mouth by a jaw clamp. Changes in convulsive threshold were measured in terms of voltage, using a calibrated Harvard inductorium.

Antagonism of metrazol, strychnine, picrotoxin, thujone, cocaine and procaine by tridione. The experiments on the antagonism of convulsant drugs by tridione in mice are summarized in table 1.

Metrazol. Convulsions were produced in all mice when 100 mg./kg. were given subcutaneously. The seizures were extreme and 7 out of 10 animals died within 30 minutes after the injection. A dose of 125 mg./kg. of metrazol subcutaneously resulted in violent seizures with all animals dying within 5 minutes after injection. A group of mice were given 500 mg./kg. tridione intraperitoneally, a dose which has little or no effect on normal activity and is approximately $\frac{1}{5}$ of the l.d.₅₀. Five to ten minutes later, 100 mg./kg. metrazol were injected subcutaneously. No convulsions occurred, and all mice lived. As seen in the table, similar results were obtained with the higher dosage of metrazol.

Picrotoxin. The convulsions resulting from the injection of 15 mg./kg. picrotoxin subcutaneously were also antagonized by 500 mg./kg. tridione. However, the antagonism is not as complete as shown towards metrazol, since increased excitability was still evident.

Thujone. The subcutaneous injection of 200 mg./kg. thujone in olive oil resulted in violent prolonged convulsions, with all animals dying within an hour. The intraperitoneal injection of 500 mg./kg. of tridione afforded complete protection.

Procaine and cocaine The results obtained with these drugs were less conclusive because of their marked depressive action on the respiratory center in doses necessary to induce convulsions in mice. However, it was observed that 500 mg/kg tridione would, in some cases, decrease seizures but did not prevent death resulting from respiratory depression.

Strychnine The convulsions produced by the subcutaneous injection of 15 mg/kg of strychnine were completely antagonized by tridione. However, a dose of 3 mg/kg could not be so antagonized by this dose of tridione.

Similar results were obtained in experiments with other species in regard to these antagonisms. A dose of 500 mg/kg tridione given orally or subcutaneously to two groups of three rabbits each caused some depression and a slight ataxia. Ten to thirty minutes later 15 mg/kg metrazol were injected intravenously without convulsive effect. (This dose was found to produce violent

TABLE 1
Antagonism of convulsant drugs by tridione in mice

NUMBER OF MICE	TRIDIONE INTRA PERITONEALLY	CONVULSANT	TRIDIONE SUBCUTANEOUSLY	NUMBER CONVULSED	NUMBER FATAL
	mg/kg		mg/kg		
10	0	Metrazol	100	10	7
10	0	Metrazol	125	10	10
15	500	Metrazol	100	0	0
12	500	Metrazol	125	0	0
5	250	Metrazol	100	0	0
5	250	Metrazol	125	2	2
10	0	Picrotoxin	15	10	10
10	500	Picrotoxin	15	1	0
7	0	Strychnine	15	5	5
8	500	Strychnine	15	0	0
5	500	Strychnine	30	5	5
5	0	Thujone	200	5	5
5	500	Thujone	200	0	0

convulsions in normal controls.) A dose of 250 mg/kg of tridione given subcutaneously was also protective. In three rabbits given 500 mg/kg orally, similar protection against metrazol was obtained.

In regard to procaine antagonism, it was found in three guinea pigs that the injection of 500 mg/kg tridione intraperitoneally was protective against 250 mg/kg procaine intramuscularly, a surely convulsant and fatal dose in untreated animals. These animals were markedly depressed for about one hour, then recovered rapidly.

It was found that the injection of a cat with 100 mg/kg metrazol intraperitoneally produced violent convulsions within five minutes. In another cat given 500 mg/kg tridione intraperitoneally, followed in five minutes by 120 mg/kg of metrazol intraperitoneally, some excitement was evident but no convulsions occurred. Later the animal was quiet and showed little or no ataxia.

Of interest were the observations on the rapidity of action of tridione. Fifteen

mice were injected with 100 mg./kg. metrazol subcutaneously and allowed to develop convulsions. A dose of 500 mg./kg. tridione was then given intraperitoneally or orally. The seizures were completely suppressed within two minutes, and all mice showed normal behavior. The same results were obtained against picrotoxin convulsions, although not all mice obtained complete alleviation of convulsions.

Data on the duration of action of tridione are summarized in table 2. The mice were injected with tridione and then, after definite periods of time, given convulsive doses of metrazol. The anticonvulsive action was apparent within five minutes. In thirty minutes the protective action was somewhat decreased,

TABLE 2
Duration of action of tridione

NUMBER OF MICE	DOSAGE OF TRIDIONE, INTRAPERITONEALLY	TIME BETWEEN INJECTIONS	DOSAGE OF METRAZOL, SUBCUTANEOUSLY	NUMBER CONVULSED	NUMBER FATAL
	mg./kg.	minutes	mg./kg.		
5	500	5	125	0	0
5	500	30	125	3	0
5	500	30	100	0	0
5	500	60	100	0	0
5	500	120	125	3	2
4	500	120	100	1	0

TABLE 3
Dilantin and phenobarbital as anticonvulsants

NUMBER OF MICE	ANTICONVULSANT	DOSAGE, INTRAPERITONEALLY	CONVULSANT	DOSAGE, SUBCUTANEOUSLY	NUMBER CONVULSED	NUMBER FATAL
		mg./kg.		mg./kg.		
5	Phenobarbital	50	Metrazol	100	0	0
5	Phenobarbital	50	Strychnine	1.5	0	0
5	Phenobarbital	50	Strychnine	3.0	0	0
5	Dilantin	25	Metrazol	100	5	5
5	Dilantin	50	Metrazol	100	5	4
5	Dilantin	100			5	

as shown by the appearance of convulsions with 125 mg./kg. metrazol. Protection against 100 mg./kg. was still present, however. At the end of two hours, the anticonvulsive action had definitely decreased.

Antagonism studies with dilantin and phenobarbital. In conjunction with the tests made on tridione, a series of experiments was conducted to compare it with phenobarbital and dilantin, the two most widely used antiepileptics. The experiments were conducted in the same manner as those described for tridione. The data are summarized in table 3. Phenobarbital is quite effective against metrazol and strychnine convulsions. It is of interest to note that phenobarbital is effective against 3 mg./kg. strychnine, while tridione is not. This may indi-

cate that the barbiturate has a more generalized depressant action on the central nervous system, including the spinal reflexes. The effective dose of 50 mg/kg intraperitoneally causes a slight ataxia and reduces general activity.

In contrast are the results obtained with dilantin, which showed no anti-convulsant action against metrazol. Dilantin alone, in doses of 100 mg/kg given intraperitoneally, caused marked excitement and convulsions. With 25 or 50 mg/kg doses of dilantin, no symptoms appeared. The injection of 100 mg/kg metrazol subcutaneously into mice previously given dilantin resulted in convulsions of the same violence as in those receiving metrazol alone. Similar results have been reported by Goodman and Lih (5). They found, however, that after administration of dilantin over a period of 4 to 7 days, protection against metrazol convulsions was present.

TABLE 4
Convulsive threshold in rats

RAT NUMBER	CONTROL THRESHOLD	DRUG	DOSE INTRAPERITONEALLY	THRESHOLD		VOLTAGE THRESHOLD INCREASE
				Time After Injection		
				5 minutes	30 minutes	
	volts		mg/kg	volts	volts	
1	1 000	Tridione	250	1,000	1,032	932
2	1 000	Tridione	250	1,100	1 330	330
3	809	Tridione	500		1,223	414
4	368	Tridione	500		809	441
5	575	Tridione	500		1,223	648
6	575	Tridione	500	809	1,223	648
7	809	Tridione	500		1,500	691
8	1 000	Tridione	1 000		*	
9	809	Phenobarbital	50		1 223	414
10	1 000	Phenobarbital	50		1,500	500
11	809	Dilantin	135		1,932	1,123
12	983	Dilantin	25		1 223	240

* Convulsive threshold was not attained, animal died with application of 2380 volts without a seizure appearing.

Studies of electrical convulsive threshold in rats In this series of experiments an attempt was made to study the effect of tridione, dilantin and phenobarbital on the electrical threshold at which convulsions would appear. The normal convulsive threshold was first determined by making gradual increases in the voltage before each stimulation period until convulsions appeared which would persist 5 to 15 seconds after cessation of electrical current. The change in threshold was measured in terms of the increased voltage necessary to induce a seizure after administering the drug. The period of stimulation was ten seconds, with five to ten minute intervals between electrical stimulation. In these studies only full tonic clonic convulsions were considered in determining thresholds. The results are summarized in table 4. As shown, all three drugs increased the convulsive threshold markedly. The method was not sufficiently refined to deter

mine which of the drugs was definitely *most* effective. It was noted that a dose of dilantin sufficient to raise the threshold markedly caused no depression of the animal's general activity, while tridione and phenobarbital caused some depression and ataxia. Phenobarbital gave the most pronounced sedation.

Tests with periods of stimulation of one second or less revealed some greatly reduced convulsive jerking movements at the control thresholds after dilantin and tridione. Thus it is open to argument whether the results of these experiments indicate a true raising of the convulsive threshold or rather give an index of the marked reduction of intensity and duration of seizures produced by these drugs.

DISCUSSION. The study of the antagonism of central nervous system excitants with drugs offers some possibility in analyzing their mode of action. This method, however, has its limitations when an attempt is made to interpret the data in terms of possible clinical use in epilepsy. Thus, we found dilantin in single doses has little or no antagonistic action against metrazol in mice, and, similarly, Knoefel and Lehmann (6) found no protection against strychnine and cocaine convulsions in cats. However, dilantin is effective in raising the electrical convulsive threshold, as shown by Merritt and Putnam (7) and Knoefel and Lehmann (6) in the cat, Tainter, et al. (8) in the rabbit, and by us in the rat.

The convulsions resulting from drugs are not generally considered to be characteristic of the seizures occurring in clinical cases (9). However, they have as a common feature the presence of a hyper-excitability state of some portion of the central nervous system. Thus, the mode of depression of this excitability by antagonistic drugs may be similar in both instances, although the site of action possibly differs.

A possible relation of epilepsy to the convulsions resulting from analeptics is indicated by a series of clinical tests conducted by Roismiser (10), who found that 1 to 3 cc. of a 10 per cent solution of metrazol given intravenously produced seizures in 35 out of 38 epileptic patients but not in normal individuals. This finding is analogous to the work of Dandy and Elman (11), who found that cats with healed cerebral injuries were three to seven times more sensitive to convulsions produced by thujone than were normal animals.

From a consideration of studies using electrical and chemical methods, it seems probable that dilantin affects primarily the cortex, and has less action on the lower brain centers which are considered to be involved in the convulsions arising from the analeptic drugs (12) (13). On the other hand, phenobarbital and tridione show a more generalized anticonvulsant action in that they antagonize chemically induced convulsions and are also capable of raising the electrical convulsive threshold. Since many studies of clinical convulsive states indicate that they may have subcortical origin, it seems at least a possibility that drugs having a more generalized action may be useful in such cases¹. It is worth noting that tridione possesses the unusual property of being an analgesic as well as an anticonvulsant but is not a true hypnotic drug. While mild sedation has been seen

¹Preliminary clinical reports indicate that *Tridione* has anticonvulsive action in epileptic patients.

in humans, its lack of hypnotic power is indicated by a clinical case where more than 30 grams were taken over a period of twelve hours without producing sleep. A further analysis of the mode and site of action of tridione will be reported by Goodman et al. (14).

SUMMARY

1. The marked antagonistic action of 3,5,5-trimethyloxazolidine-2,4-dione (Tridione) against convulsions produced by drugs and electrical shock has been demonstrated in experimental animals.

2. Comparative experiments with phenobarbital and dilantin revealed that tridione is more comparable to phenobarbital in its action but produces less depression in effective doses.

REFERENCES

- (1) ERLIENMEYER, *Helv. Chim. Acta*, 21: 1013 (1938).
- (2) LUTON, BLALOCK, BAXTER, JR., AND STODGTON, *Proc. Soc. Exp. Biol. Med.*, 47, 245, 1941.
- (3) SPIELMAN, *J. Am. Chem. Soc.* (In press).
- (4) RICHARDS AND EVERETT, *Federation Proc. Am. Soc. Exp. Biol.*, 3: 39, 1944.
- (5) GOODMAN AND LIN, *This Journal (Proc.)*, 72: 18, 1941.
- (6) KNOEFEL AND LEHMANN, *This Journal*, 76: 194, 1942.
- (7) MERRITT AND PUTNAM, *Arch. Neurol. Psychiatry*, 39: 1003, 1938.
- (8) TAINTER, TAINTER, LAWRENCE, NEURD, LACKEY, LUDUENA, KIRTLAND, JR., AND GONZALEZ, *This Journal*, 79: 42, 1943.
- (9) LENNOX, NELSON, AND BEETHAM, *Arch. Neurol. Psychiatry*, 21: 625, 1929.
- (10) ROISMISER, *Semana Medica*, Buenos Aires, 60: 310, 1943.
- (11) DANDY AND ELMAN, *J. Hop. Bull.*, 38: 40, 1925.
- (12) HILDEBRANDT, *Handbuch der Exp. Pharm.*, 5: 151, 1937.
- (13) SCHRIEVER AND PERSCHMANN, *C. R. de la Soc. de Biol.*, 120: 971, 1935.
- (14) GOODMAN, MANUEL, AND BAUM, (Manuscript in preparation).

INDEX

- Ahreu, B E, Harper, H T, Jr, Woodhury, R A, Hamilton, W F, and Volpitta, Perry P Cardiac and blood pressure effects of pitocin (oxytocin) in man, 95
- Adrenolytic and sympatholytic actions of yohimbine and ethyl yohimbine, 111
- Ahlquist, Raymond P Contribution to pharmacology of aliphatic amines, 235
- Allantoin in mammals, Absorption and excretion of, 1
Role of pyrogens in alleged leukocytic response to, 10
- Aliphatic amines, Contribution to pharmacology of, 235
- Allmark, M, and Bliss, C I Digitalis cat assay in relation to rate of injection, 378
- Alloxazine adenine dinucleotide and cozymase, Destruction of, in tissues during shock, 164
- Amides, amines and related compounds as diuretics, 84
- Amide-substituted phenyl arsenoxides and their derivatives, toxicity and treponemicidal activity of, 142
- Amino acid oxidase and lactic dehydrogenase, Inactivation of apoenzyme of, in anoxia, 240
- Anaesthesia, Changes in activity of pulmonary receptors in, and their influence on respiratory behaviour, 310
- Andrews, Howard L, and Himmelsbach, C K Relation of intensity of morphine abstinence syndrome to dosage, 288
- Anemia, anti-pernicious, activity, Bone marrow procedure for assay of liver extracts for, 248
- Anesthesia, spinal, in experimental animals, Local nervous tissue changes following, 209
- Anesthetic activity of *cis-trans* isomers of trichloroethylidene glycerol, 72
- Anoxemic effects, Comparative, from carbon monoxide hemoglobin and methemoglobin, 182
- Anoxia, Inactivation of apoenzyme of amino acid oxidase and lactic dehydrogenase in, 240
- Anticonvulsive action of drugs 402
- Antimonials, some organic, Toxicity and trypanocidal activity of, 224
- Atabrine, rational use of, in treatment of malaria, Pharmacological basis for, 307
- Azide and sulfanilamide, Effects of, on oxygen consumption and cell division in egg of sea urchin, *Arbacia punctulata*, 58
- Badger, Elizabeth A, Schmidt, Ida G, Schmidt, L H, and Hughes, Hettie B Toxicity of sulfamerazine and sulfamethazine, 17
- Barbiturate hypnosis, Evaluation of influence of succinate and malonate on, 203
- Barcham, I, Nevin, Marshall I, Co Tui und Preiss, A L Local nervous tissue changes following spinal anaesthesia in experimental animals, 209
- Benson, Wilbur M, Koelle, Ethel S, and Mattis, Paul A Toxicological studies of phthalylsulfathiazole, 116
- Berliner, Robert W, Shannon, James A, Earle, David P, Jr, Brodic, Bernard B, and Taggart, John V Pharmacological basis for rational use of atabrine in treatment of malaria, 307
- Bernheim, Frederick, and Bernheim, Mary L C Oxidation in vitro of morphine by rat liver slices, 374
- Bernheim, Mary L C, and Bernheim, Frederick Oxidation in vitro of morphine by rat liver slices, 374
- Bett, H D, and Young, C M Bone marrow procedure for assay of liver extracts for anti-pernicious anemia activity, 248
- Beyer, Karl H, and Latven, Albert R Evaluation of influence of succinate and malonate on barbiturate hypnosis, 203
- Bigelow, Nolton, and Harrison, Irving General analgesic effects of procaine, 368
- Bliss C I, and Allmark, M G Digitalis cat assay in relation to rate of injection, 378
- Blood, Iodine in, and thyroid, 331
pressure and cardiac effects of pitocin (oxytocin) in man, 95

- Bone marrow procedure for assay of liver extracts for anti-pernicious anemia activity, 248
- Brodie, Bernard B., Taggart, John V., Berliner, Robert W., Shannon, James A., and Earle, David P., Jr. Pharmacological basis for rational use of atabrine in treatment of malaria, 307
- Brooks, G. W., Unna, K., and Pick, E. P. Inhibitory effect of sulfonamides on action of nicotine in isolated intestine, 133
- Bülbring, E., and Whitteridge, D. Changes in activity of pulmonary receptors in anaesthesia and their influence on respiratory behaviour, 340
- Butler, Thomas C. Anesthetic activity of *cis-trans* isomers of trichloroethylidene glycerol, 72
- t*-Butyl hydrogen peroxide and simple unsaturated lactones, Effect of, on isolated frog heart, 151
- Cameron, W. M., Whitsell, L. J., Hartman, M. M., and Tainter, M. L. Clinical actions of ethylnorsuprarenin, 269
- Cantoni, G. L., and Loewi, O. Inhibition of cholinesterase activity of nervous tissues by eserine in vivo, 67
- Carbon monoxide hemoglobin and methemoglobin, Comparative anoxic effects from, 182
- Cardiac activity, substances with, Pharmacology and chemistry of, 151 and blood pressure effects of pitocin (oxytocin) in man, 95
- Cartland, George F., and Grabam, Boyd E. Comparative pharmacological actions of beta-hydroxy and methoxy phenyl-n-propylamines, 360
- Cell division and oxygen consumption, Effects on, of sulfanilamide and azide, in egg of sea urchin, *Arbacia punctulata*, 58
- Chan, L. K., and Way, E. Leong. Toxicity and trypanocidal activity of p-sulfonamidophenylarsonic acid and certain of its derivatives, 278
- Chase, Harold F., Lehman, Arnold J., and Yonkman, Frederick F. Antispasmodic activity of some 4-morpholinealkyl esters, 174
- Cholinesterase activity, Inhibition of, in nervous tissues by eserine in vivo, 67
- Cinchona bark, Plasma concentrations following oral administration of single doses of principal alkaloids of, 160
- Clark, William G., Strakosch, Ernest A., and Cranston, Elizabeth M. Acute toxicity for mice of "mapharsen" and sodium sulfathiazole administered separately and in combination, 284
- Co Tui, Preiss, A. L., Barcham, I., and Nevin, Marshall I. Local nervous tissue changes following spinal anesthesia in experimental animals, 209
- Cozymase and alloxazine adenine dinucleotide, Destruction of, in tissues during shock, 164
- Cranston, Elizabeth M., Clark, William G., and Strakosch, Ernest A. Acute toxicity for mice of "mapharsen" and sodium sulfathiazole administered separately and in combination, 284
- Culbertson, James T., and Rose, Harry M. Chemotherapy of filariasis in cotton rat by administration of neostam and neostibosan, 189
- Cyclopropane anesthesia, ventricular tachycardia during, Relationship to, of chemical structure of sympathomimetic amines, 197
- Digitalis cat assay in relation to rate of injection, 378
- Diuretics, Amides, amines and related compounds as, 84
- Doak, George O., Steinman, Harry G., Eagle, Harry, and Hogan, Ralph B. Toxicity and treponemocidal activity of amide-substituted phenyl arsenoxides and their derivatives, 142
- Eagle, Harry, Hogan, Ralph B., Doak, George O., and Steinman, Harry G. Toxicity and treponemocidal activity of amide-substituted phenyl arsenoxides and their derivatives, 142
- Earle, David P., Jr., Brodie, Bernard B., Taggart, John V., Berliner, Robert W., and Shannon, James A. Pharmacological basis for rational use of atabrine in malaria, 307
- Ellis, Fred W., Freedman, Goldie, and Gruber, Charles M. Toxicological and pharmacological investigation of sodium sec-butyl ethyl barbituric acid (butisol sodium), 254

- Eserine, Inhibition by, of cholinesterase activity in nervous tissues *in vivo*, 67
- Ethylorsuprenin, Clinical actions of, 269
- Everett, Guy M., and Richards, Richard A. Comparative anticonvulsant action of 3,5,5-trimethylloxazolidine 2,4 dione (Tridione), dilantin and phenobarbital, 402
- Filariasis in cotton rat, Chemotherapy of, by administration of neostam and neostibosan, 189
- Fisher, Kenneth C., Henry, R. J., and Low, E. Effects of sulfanilamide and azide on oxygen consumption and cell division in egg of sea urchin, *Arbacia punctulata*, 58
- Freedman, Goldie, Gruber, Charles M., and Ellis, Fred W. Toxicological and pharmacological investigation of sodium sec-butyl ethyl barbituric acid (butisol sodium), 254
- Goldberg, M. L., Wakerlin, G. E., Johnson, C. A., and Moss, W. G. Treatment of experimental renal hypertension with renal extracts, 101
- Goodwin, L. G. Toxicity and trypanocidal activity of some organic antimonials, 224
- Graham, Boyd E., and Cartland, George F. Comparative pharmacological actions of beta hydroxy and methoxy phenyl-n-propylamines, 360
- Greenberg, Leon A., and Lester, David. Comparative anoxic effects from carbon monoxide hemoglobin and methemoglobin, 182
- Greig, Margaret E. Studies on shock induced by hemorrhage. VII. Destruction of cozymase and alloxazine adenine dinucleotide in tissues during shock, 164
- VIII. Inactivation of apoenzyme of amino acid oxidase and lactic dehydrogenase in anoxia, 240
- Gruber, Charles M., Ellis, Fred W., and Freedman, Goldie. Toxicological and pharmacological investigation of sodium sec butyl ethyl barbituric acid (butisol sodium), 254
- Hadidian, Zareh, and Lipschutz, Werner L. Amides, amines and related compounds as diuretics, 84
- Halperin, N., Salter, W. T., and Sappington, T. S. Indine in blood and thyroid. VII. Analytical procedure for use in small samples pharmacological range of concentrations, 331
- Hamilton, W. F., Volpitta, Perry P., Abreu, B. E., Harper, H. T., Jr., and Woodbury, R. A. Cardiac and blood pressure effects of pitocin (oxytocin) in man, 95
- Harrison, Irving and Bigelow, Nolton. General analgesic effects of procaine, 368
- Hartman, M. M., Tainter, M. L., Cameron, W. M., and Whitsell, L. J. Clinical actions of ethylorsuprenin, 269
- Hawkins, W. W. and Young E. Gordon. Role of pyrogens in alleged leukocytic response to allantoin, 10
- Young, E. Gordon, and Wentworth, Helen P. Absorption and excretion of allantoin in
- Heart, isolated frog. Effect on, of simple unsaturated lactones and *t*-butyl hydrogen peroxide, 151
- Hemolytic anemia in mice, Comparative activity of sulfamerazine, sulfadiazine and sulfapyridine in production of, 301
- Hemorrhage, Studies on shock induced by, 164, 240
- Henry, R. J., Low, E., and Fisher, Kenneth C. Effects of sulfanilamide and azide on oxygen consumption and cell division in egg of sea urchin, *Arbacia punctulata*, 58
- Huatt, Edwin P. Plasma concentrations following oral administration of single doses of principal alkaloids of cinchona bark, 160
- Himmelsbach, C. K., and Andrews, Howard L. Relation of intensity of morphine abstinence syndrome to dosage, 283
- Hitebings, George H., and Randall, Lowell O. Effect of tyrosinase on phenethylamine derivatives, 77
- Hogan, Ralph B., Doak, George O., Steinman, Harry G., and Eagle, Harry. Toxicity and treponemicidal activity of amide-substituted phenyl arsenoxides and their derivatives, 142
- Hughes, Hettie B., Badger, Elizabeth A., Schmidt, Ida G., and Schmidt, L. H. Toxicity of sulfamerazine and sulfamethazine, 17

- Schmidt, L. H., and Scsler, Clara L. Chemotherapeutic activities of sulfamerazine and sulfamethazine, 43
- Hypertension, experimental renal, Treatment of, with renal extracts, 101
- Hypnosis, barbiturate, Evaluation of influence of succinate and malonate on, 203
- Iodine in blood and thyroid, 331
- Jelinek, Viola C., and Scudi, John V. Distribution method for differentiation of urinary excretion products of sulfonamides; role of these products in urolithiasis, 218
- Jeremias, Robert, Yonkman, Fredrick F., and Stilwell, Don. Adrenolytic and sympatholytic actions of yohimbine and ethyl yohimbine, 111
- Johnson, C. A., Moss, W. G., Goldberg, M. L., and Wakerlin, G. E. Treatment of experimental renal hypertension with renal extracts, 101
- Koelle, Ethel S., Mattis, Paul A., and Benson, Wilbur M. Toxicological studies of phthalylsulfathiazole, 116
- Lactic dehydrogenase and amino acid oxidase, Inactivation of apoenzyme of, in anoxia, 240
- Lactones, simple unsaturated, and *t*-butyl hydrogen peroxide, Effect of, on isolated frog heart, 151
- Latven, Albert R., and Beyer, Karl H. Evaluation of influence of succinate and malonate on barbiturate hypnosis, 203
- and Welch, Arnold D. Sulfamerazine. III. Comparative activity of sulfamerazine, sulfadiazine and sulfapyridine in production of hemolytic anemia in mouse, 301
- Lehman, Arnold J., Yonkman, Fredrick F., and Chase, Harold F. Antispasmodic activity of some 4-morpholinealkyl esters, 174
- Lester, David, and Greenberg, Leon A. Comparative anoxemic effects from carbon monoxide hemoglobin and methemoglobin, 182
- Leukocytic response, alleged to allantoin, Role of pyrogens in, 10
- Lipschitz, Werner L., and Hadidian, Zareh. Amides, amines and related compounds as diuretics, 84
- Liver extracts for anti-pernicious anemia activity, Bone marrow procedure for assay of, 248
- Loewi, O., and Cantoni, G. L. Inhibition of cholinesterase activity of nervous tissues by eserine *in vivo*, 67
- Low, E., Fisher, Kenneth C., and Henry, R. J. Effects of sulfanilamide and azide on oxygen consumption and cell division in egg of sea urchin, *Arbacia punctulata*, 58
- Malaria, rational use of atabrine in treatment of, Pharmacological basis for, 307
- Malonate and succinate, Evaluation of influence of, on barbiturate hypnosis, 203
- "Mapharsen" and sodium sulfathiazole, Acute toxicity for mice of, administered separately and in combination, 234
- Mattis, Paul A., Benson, Wilbur M., and Koelle, Ethel S. Toxicological studies of phthalylsulfathiazole, 116
- Meek, Walter J., Orth, O. S., and Stutzman, J. W. Relationship of chemical structure of sympathomimetic amines to ventricular tachycardia during cyclopropane anesthesia, 197
- Mendez, Rafael. Pharmacology and chemistry of substances with cardiac activity. III. Effect of simple unsaturated lactones and *t*-butyl hydrogen peroxide on isolated frog heart, 151
- Methemoglobin and carbon monoxide hemoglobin, Comparative anoxemic effects from, 182
- Morphine abstinence syndrome, Relation of intensity of, to dosage, 238
- Oxidation *in vitro* of, by rat liver slices, 374
- 4-Morpholinealkyl esters, Antispasmodic activity of, 174
- Moss, W. G., Goldberg, M. L., Wakerlin, G. E., and Johnson, C. A. Treatment of experimental renal hypertension with renal extracts, 101
- Neostam and neostibosan, administration of, Chemotherapy of filariasis in cotton rat by, 189

- Neostihosan and neostam, Chemotherapy of filariasis in cotton rat by administration of, 189
- Nervous tissue changes, Local, following spinal anesthesia in experimental animals, 209
transmission in synapses and end plates, Inhibition of, by thiamine, 294
- Nevin, Marshall I, Co Tui, Preiss, A L, and Barcham, I Local nervous tissue changes following spinal anesthesia in experimental animals, 209
- Nicotine, action of, in isolated intestine, Inhibitory effect of sulfonamides on, 133
- Orth, O S, Stutzman, J W, and Meek, Walter J Relationship of chemical structure of sympathomimetic amines to ventricular tachycardia during cyclopropane anesthesia, 197
- Oxygen consumption and cell division, Effects on, of sulfanilamide and azide, in egg of sea urchin, *Arbacia punctulata*, 58
- Pharmacology of aliphatic amines, Contribution to, 235
- Phenethylamine derivatives, Effect on, of tyrosinase, 77
- Phthalylsulfthiazole, Toxicological studies of, 116
- Pick, E P, and Unna, K Inhibition of nervous transmission in synapses and end plates by thiamine, 294
- Brooks, G W, and Unna, K Inhibitory effect of sulfonamides on action of nicotine in isolated intestine, 133
- Pitocin (oxytocin), Cardiac and blood pressure effects of, in man, 95
- Plasma concentrations following oral administration of single doses of principal alkaloids of cinchona bark, 160
- Preiss, A L, Barcham, I, Nevin, Marshall I, and Co Tui Local nervous tissue changes following spinal anesthesia in experimental animals, 209
- Procaine, General analgesic effects of, 368
- Propylamines, beta hydroxy and methoxy phenyl n-, Comparative pharmacological actions of, 360
- Pulmonary receptors, Changes in activity of, in anaesthesia and their influence on respiratory behaviour, 340
- Pyrogens, Role of, in alleged leukocyte response to allantoin, 10
- Randall, Lowell O, and Hitchings, George H Effect of tyrosinase on phenethylamine derivatives, 77
- Renal extracts, Treatment with, of experimental renal hypertension, 101
- Respiratory behaviour, Changes in activity of pulmonary receptors in anaesthesia and their influence on, 340
- Richards, Richard K, and Everett, Guy M Comparative anticonvulsant action of 3,5,5 - trimethylxazolidine - 2,4-dione (Tridione), dilaatin and phenobarbital, 402
- Robinson, Harry J, and Smith, Dorothy G Streptothricin as chemotherapeutic agent, 390
- Rose, Harry M, and Culbertson, James T Chemotherapy of filariasis in cotton rat by administration of neostam and neostihosan, 189
- Salter, W T, Sappington, T S, and Halperin, N Iodins in blood and thyroid VII Analytical procedure for use in small samples pharmacological range of concentrations, 331
- Sappington, T S, Halperin, N, and Salter, W T Iodine in blood and thyroid VII Analytical procedure for use in small samples pharmacological range of concentrations, 331
- Schmidt, Ida G, Schmidt, L H, Hughes, Hettie B, and Badger, Elizabeth A Toxicity of sulfamerazine and sulfamethazine, 17
- Schmidt, L H, Hughes, Hettie B, Badger, Elizabeth A, and Schmidt, Ida G Toxicity of sulfamerazine and sulfamethazine, 17
- Sesler, Clara L, and Hughes, Hettie B Chemotherapeutic activities of sulfamerazine and sulfamethazine, 43
- Scudi, John V, and Jelinek, Viola C Distribution method for differentiation of urinary excretion products of sulfonamides, role of these products in urolithiasis, 218
- Sea urchin, *Arbacia punctulata* egg, of, oxygen consumption and cell division in, Effects on, of sulfanilamide and azide, 58
- Sesler, Clara L, Hughes, Hettie B, and Schmidt, L H Chemotherapeutic activities of sulfamerazine and sulfamethazine, 43

- Shannon, James A., Earle, David P., Jr., Brodie, Bernard B., Taggart, John V., and Berliner, Robert W. Pharmacological basis for rational use of atahrine in treatment of malaria, 307
- Shock, Destruction of cozymase and alloxazine adenine dinucleotide in tissues during, 164
- induced by hemorrhage, Studies on, 164, 240
- Smith, Dorothy G., and Robinson, Harry J. Streptothricin as chemotherapeutic agent, 390
- Sodium sec-butyl ethyl harbituric acid (butisol sodium), Toxicological and pharmacological investigation of, 254
- sulfathiazole and "mapharsen," Acute toxicity for mice of, administered separately and in combination, 284
- Spinal anesthesia in experimental animals, Local nervous tissue changes following, 209
- Steinman, Harry G., Eagle, Harry, Hogan, Ralph B., and Doak, George O. Toxicity and treponemicidal activity of amide-substituted phenyl arsenoxides and their derivatives, 142
- Stilwell, Don, Jeremias, Robert, and Yonkman, Fredrick F. Adrenolytic and sympatholytic actions of yohimbine and ethyl yohimbine, 111
- Strakosch, Ernest A., Cranston, Elizabeth M., and Clark, William G. Acute toxicity for mice of "mapharsen" and sodium sulfathiazole administered separately and in combination, 284
- Streptothricin as chemotherapeutic agent, 390
- Stutzman, J. W., Meek, Walter J., and Orth, O. S. Relationship of chemical structure of sympathomimetic amines to ventricular tachycardia during cyclopropane anesthesia, 197
- Succinate and malonate, Evaluation of influence of, on barbiturate hypnosis, 203
- Sulfadiazine, sulfapyridine and sulfamerazine, Comparative activity of, in production of hemolytic anemia in mouse, 301
- Sulfamerazine, Chemotherapeutic activities of, 43
- sulfadiazine and sulfapyridine, Comparative activity of, in production of hemolytic anemia in mouse, 301
- Toxicity of, 17
- Sulfamethazine, Chemotherapeutic activities of, 43
- Toxicity, of, 17
- Sulfanilamide and azide, Effects of, on oxygen consumption and cell division in egg of sea urchin, *Arbacia punctulata*, 53
- Sulfapyridine, sulfamerazine and sulfadiazine, Comparative activity of, in production of hemolytic anemia in mouse, 301
- Sulfonamides, Inhibitory effect of, on action of nicotine in isolated intestine, 133
- Urinary excretion products of, Distribution method for differentiation of; role of these products in urolithiasis, 218
- p-Sulfonamidophenylarsonic acid and certain of its derivatives, Toxicity and trypanocidal activity of, 278
- Sympatholytic and adrenolytic actions of yohimbine and ethyl yohimbine, 111
- Sympathomimetic amines; Relationship of chemical structure, to ventricular tachycardia during cyclopropane anesthesia, 197
- Tachycardia, ventricular, during cyclopropane anesthesia, Relationship of chemical structure of sympathomimetic amines to, 197
- Taggart, John V., Berliner, Robert W., Shannon, James A., Earle, David P., Jr., and Brodie, Bernard B. Pharmacological basis for rational use of atahrine in treatment of malaria, 307
- Tainter, M. L., Cameron, W. M., Whitsell, L. J., and Hartman, M. M. Clinical actions of ethylnorsuprarenin, 269
- Thiamine, Inhibition of nervous transmission in synapses and end plates by, 294
- Thyroid and iodine in blood, 331
- Toxicity, Acute, for mice; of "mapharsen" and sodium sulfathiazole administered separately and in combination, 284
- and trypanocidal activity of p-sulfonamidophenylarsonic acid and certain of its derivatives, 278
- and trypanocidal activity of some organic antimonials, 224
- Trichloroethylidene glycerol, Anesthetic activity of *cis-trans* isomers of, 72
- Trypanocidal activity and toxicity of p-sulfonamidophenylarsonic acid and certain of its derivatives, 278

- activity and toxicity of some organic antimonials, 224
- Tyrosinase, Effect of, on phenethylamine derivatives, 77
- Unna, K, and Pick, E P Inhibition of nervous transmission in synapses and end plates by thiamine, 294
- Pick, E P, and Brooks, G W Inhibitory effect of sulfonamides on action of nicotine in isolated intestine, 133
- Urinary excretion products of sulfonamides, Distribution method for differentiation of, role of these products in urolithiasis, 218
- Urolithiasis, Role in, of urinary excretion products of sulfonamides, 218
- Volpitto, Perry P, Abreu, B E, Harper, H T, Jr, Woodbury, R A, and Hamilton, W F Cardiac and blood pressure effects of pitocin (oxytocin) in man, 95
- Wakerlo, G E, Johnson, C A, Moss, W G, and Goldberg, M L Treatment of experimental renal hypertension with renal extracts, 101
- Way, E Leong, and Chan, L K Toxicity and trypanocidal activity of p-sulfonamidophenylarsoic acid and certain of its derivatives, 278
- Welch, Arnold D, and Latven, Albert R Sulfamerazine III Comparative activity of sulfamerazine, sulfadiazine and sulfapyridine in production of hemolytic anemia in mouse 301
- Wentworth, Helen P, Hawkins, W W, and Young, E Gordon Absorption and excretion of allantoin in mammals, 1
- Whitsell L J, Hartman, M M, Tainter, M L, and Cameron, W M Clinical actions of ethylnorsuprarenin, 269
- Whittridge, D, and Bülbring, E Changes in activity of pulmonary receptors in anaesthesia and their influence on respiratory behaviour, 340
- Woodbury, R A, Hamilton, W F, Volpitto, Perry P, Abreu, B E, and Harper, H T, Jr Cardiac and blood pressure effects of pitocin (oxytocin) in man, 95
- Yohimbine and ethyl yohimbine, Adrenolytic and sympatholytic actions of, 111
- Yonkman, Fredrick F, Chase, Harold F, and Lehman, Arnold J Antispasmodic activity of some 4-morpholinealkyl esters, 174
- Stilwell, Don, and Jeremias, Robert Adrenolytic and sympatholytic actions of yohimbine and ethyl yohimbine, 111
- Young, C M, and Bett, H D Bone marrow procedure for assay of liver extracts for anti pernicious anemia activity, 243
- Young, E Gordon, and Hawkins, W W Role of pyrogens in alleged leukocytosis response to allantoin, 10
- Wentworth, Helen P, and Hawkins, W W Absorption and excretion of allantoin in mammals, 1